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FAILURE OF *CANDIDA STELLATOIDEA* TO QUALIFY AS A SPECIES

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Candida stellatoidea (Langeron and Guerra) has been classically differentiated from *Candida albicans* (Berkhout) by failure of the former to produce acid from sucrose, lack of pathogenicity for rabbits, and delayed production of chlamydospores (Lodder and Kreger-Van Rij, 1952). In the course of a carrier study* among patients of the Tumor Therapy Clinic of the Children's Cancer Research Foundation, many strains of candida have been isolated which do not satisfy the usual criteria for either species; thus a detailed comparison of these with a number of more typical strains was undertaken.

METHODS

Differential fermentation tests were performed as recommended by Martin et al (1937 and 1940), using 1.0% carbohydrate in vaseline-sealed tubes of brom-thymol-blue broth. All strains were verified as *Candida* by slide culture, and chlamydospore production was induced in the medium described by Nickerson and Mankowski (1953). Rabbit inoculation was done in some instances.

RESULTS

One hundred and ninety-five strains which produced acid and gas from glucose and maltose but failed to produce gas in sucrose were selected for study from more than 500 such strains isolated from the mouth or internal viscera of patients who consistently harbored *Candida* of this fermentation type in the mouth. A small number of these strains unequivocally produced acid from sucrose (i.e., the indicator changed to a clear yellow), a large number did not visibly change the indicator, but the majority fell within a wide range of color changes, varying from a slight greenish-blue to a more pronounced yellowish green. Since it was impossible to distinguish between *albicans* and *stellatoidea* on this basis, 162 strains were rechecked simultaneously, with subjective color readings made at regular intervals for 14 days. As indicated in table 1, only 3% of these strains were active acid producers, 25% produced minimal or no change in the indicator, and the remainder fell somewhere in between. These groups were artificial and arbitrary, as evidenced by the failure of two observers to agree as to how a given strain should be grouped.

The biochemical definition of these species uses the word "acid" without defining how much acid; therefore a more quantitative measure of acid pro-

TABLE 1.—*Incidence of degrees of acid production (from sucrose) among 162 strains of* Candida.*

Arbitrary degree† of acid production	Number of strains	Percent
Vigorous	4	3
Weak	117	72
Very weak	41	25

* As defined in the text.
† Based on rough grouping of observed indicator changes.

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* Results to be published elsewhere.

duction was sought. The addition of 0.1 *N* hydrochloric, lactic, or citric acid to the carbohydrate medium produced a constant straight-line relationship between milliequivalents of acid and pH (potentiometric); thus, the medium showed no significant buffering ability. Hence it was felt that pH change reliably measured acid production, irrespective of the nature of the acid produced.

The potentiometric method was applied to 35 strains of *C. albicans* and *C. stellatoidea* previously isolated and classified in these laboratories or obtained as stock cultures.† Because of the statement of Martin et al (1937) regarding the importance of an initial pH of 7.2, viz., "an increase of 0.2 pH will inhibit the fermentation of sucrose by *C. albicans*," acid production was compared in medium adjusted to an initial pH of 7.9, 7.6, 7.3, and 7.0. Back titration to the initial pH with *N*/10 sodium hydroxide was employed in several instances to check further the potentiometric determination. All cultures were incubated 7 days.

The results, as illustrated in table 2 with one strain of *Candida*, indicate that the two methods of acidimetry are in reasonable agreement. Acid production was not only undiminished when the initial pH was 7.9, but was slightly greater than at a lower initial pH. This was true, without exception, of the 36 strains so tested. The first visible change in the brom-thymol-blue indicator occurs at about pH 7.2 to 7.0, and it is difficult to separate visually any color differences caused by changes of less than 0.2 to 0.3 pH units. Therefore, the statement of Martin et al (1937) should be revised to read "the amount of acid produced from sucrose by some strains of *C. albicans* is so small that no discernible color change is produced at an initial pH above 7.2."

TABLE 2.—*Acid production by a strain of* C. stellatoidea* *in sucrose medium with varied initial pH.*

Initial pH	Final pH	Meq acid/ml, measured by	
		pH meter	NaOH titration
7.9	7.2	0.0030	0.0032
7.6	6.7	0.0036	0.0035
7.3	6.8	0.0024	0.0032
7.0	6.5	0.0022	0.0027

* Strain no. 2397, Instituto Oswaldo Cruz.

Table 3 summarizes the acid production from sucrose of 8 strains in medium with an initial pH of 7.0. The smallest change noted, 0.0004 meq/ml, represents a pH change of 0.1, which is not within the range of reliable visual differentiation. The 35 strains ranged from this level up to 0.0054 meq/ml, which represented a pH change of 1.2 units, with development of a complete change in the indicator.

The ability to produce chlamydospores is included in the definition of *C. albicans* and *C. stellatoidea* alike, but the ease with which these special structures are produced has been used as a differential point for species characterization (Gordon et al, 1952) and estimation of pathogenicity. Chlamydospore production was studied in thirty strains for comparison with biochemical reactions and rabbit pathogenicity. Different strains varied markedly with respect to number of chlamydospores and rapidity of development, and there was a complete lack of correlation with other diagnostic characteristics. Certain strains which had produced fatal human disease produced no chlamydospores and did not produce acid from sucrose.

A further differential criterion (Martin et al, 1940) is the reported patho-

† We wish to express our appreciation to Dr. Rhoda Benham, College of Physicians and Surgeons, Columbia University, New York City, Dr. A. E. de Area Leão, Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, and Dr. J. Lodder, Koninklijke Gist-en Spiritusfabriek, Delft, Nederlands, for providing a number of the stock strains which were used in this study.

TABLE 3.—*Acid production from sucrose by various strains of* C. albicans *and* C. stellatoidea: *relation to pathogenicity.*

Strain*	Species	Acid production, meq/ml	Rabbit pathogenicity	
			Renal lesions	Outcome
ATCC-10231	*albicans*	0.0045	Many, small	Died, 4 days
RB-1887	*stellatoidea*	0.0018	None	Sacrificed, 10 days
CCRF-60115		0.0014	Moderate, larger	Died, 6 days
CCRF-60116		0.0014	Rare, small	Sacrificed, 10 days
CCRF-60112		0.0009	Many, small	Died, 4 days
ATCC-11006	*stellatoidea*	0.0009	None	Sacrificed, 10 days
CCRF-60113		0.0004	Rare, small	Sacrificed, 10 days
CBS-1	*stelloidea*	0.0004	None	Sacrificed, 10 days

* ATCC: American Type Culture Collection, strain 10,231; RB: Dr. Rhoda Benham; CCRF: Children's Cancer Research Foundation; CBS: Centraalbureau voor Schimmelcultures, Delft, Nederlands, received from Dr. J. Lodder.

genicity for rabbits of *C. albicans* and the lack of this property in *C. stellatoidea*. Fifteen strains were tested by the intravenous inoculation of 1.0 ml of a 1.0% suspension of blastospores into rabbits weighing 2.0 to 2.5 kg. The results obtained with eight representative strains are summarized in table 3. Correlation is lacking between vigor of acid production and ability of the organism to produce renal lesions. Thus, some strains clearly classifiable as *C. stellatoidea* on the basis of acid production would be called *C. albicans* on the basis of rabbit pathogenicity.

DISCUSSION

Martin et al (1937 and 1940), by pointing out that the great majority of freshly isolated strains of *Candida* could be classified as one or another of six species, have simplified the taxonomy of this genus, at least for the medical mycologist. However, the fact that *C. stellatoidea* was first considered as a variety of *C. albicans* rather than a distinct species, implies that the differentiation may not always be clear.

When the defining characteristics are examined critically, none is found to be entirely reliable. Sucrose fermentation, as illustrated by the present studies, shows a wide range of variation. Gross colonial morphology as observed in these laboratories has been unreliable, *C. albicans* sometimes producing irregular colonies, while those of *C. stellatoidea* frequently are entire. Similar observations have been made by Conant (1953). The microscopic structure of the mycelium varies considerably from strain to strain and is not diagnostic; both species produce chlamydospores at a rate and to a degree which varies with different strains. This was emphasized by Gordon et al (1952), who defined *C. albicans* only on the basis of rapid chlamydospore formation, citing two strains of *albicans* with the fermentation characteristics of *stellatoidea*. Rabbit pathogenicity has been considered above; here also there is too much variation to permit specific distinction. *C. stellatoidea* has been reported to occur only in the vagina, but 41 strains isolated in these laboratories from cultures (postmortem) of the mouth or internal viscera (Craig and Farber, 1953) fulfill the diagnostic criteria for that species. The production of green pigment by *C. stellatoidea* (Jones and Peck, 1940) has not been examined in detail.

The possible role of dissociation in this problem should be considered. MacKinnon (1940 and 1945) and George and Plunkett (1948) studied spontaneous dissociation in a rough or membranous direction, and Mickle and Jones (1940) have induced similar dissociation by use of lithium chloride or specific antiserum. These reports indicate that dissociation is not necessarily abrupt and complete, but rather is a direction of change, that at least partial reversion

can occur, that dissociation may be accompanied by a proportionate loss of rabbit pathogenicity, and that the biochemical characteristics remain generally unchanged. Ranque and Depieds (1952), in contrast, observed a diminished ability to ferment galactose, levulose, and maltose in a number of rough variants of *C. albicans*, which was reversible as the variant reverted towards the smooth form. Disssociation is not merely a function of laboratory manipulation however, since deMenna (1952) recorded rough variants on primary isolation (*C. albicans*, 4 strains), as did Castellani in 1938 (*C. tropicalis*, 2 strains); and a number of naturally occurring rough variants, some of which reverted to smooth after a few transfers, also were isolated during the present studies. One can only speculate as to the possibility that the observed differences between *C. albicans* and *C. stellatoidea* are dissociative in nature, but it is interesting that the differences in morphology and pathogenicity are compatible with such a view.

Regardless of the fundamental reason for the variation demonstrated by the present studies, it is clear that *C. albicans* and *C. stellatoidea* are indeed merely the extremes of a broad spectrum of variation and should not be separated into species. Instead, the definition of the species *C. albicans* should be altered to the effect that "the organism produces acid from sucrose weakly, without gas, and is pathogenic for rabbits, either or both characteristics being variable to the point of disappearance."

SUMMARY

A study of 195 strains of *Candida* showing a complete range of variation from the characters considered diagnostic of *C. albicans* to those diagnostic of *C. stellatoidea* is presented, with emphasis on biochemical reactions as well as on morphology and rabbit pathogenicity. No justification was found for separating these strains into two species.

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THE EFFECTS OF HYPOXIA ON INFECTIONS WITH *PLASMODIUM CATHEMERIUM*

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Latent experimental malarial infections in canaries, with *Plasmodium cathemerium*, can readily be made patent and visible in stained blood films by subjection of the birds to an adequate stress, such as exposure to hypoxic atmospheres, as reported from this laboratory in 1944.[1] It was also reported at that time that when the infections had run their full courses to latency without the intervention of antimalarial drugs, the infections were less readily relapsed than were the infections in birds that had been treated by drugs in such a way as to reduce the severity of the infection. Thus it is evident that the readiness to relapse may be considered to serve as an indication of the grade or severity of the course of the primary infection. The more severe the infection, if the animal survives, the greater or more effective are the protective mechanisms which in these conditions involve primarily phagocytic processes, and conversely, the less severe the infection, the less the development of these defense reactions.

Such studies in a rather general way, therefore, reveal to what extent chemotherapeutic effects of antimalarial drugs depend upon the defensive phagocytic and other capacities of the host. These considerations led us to the study of the effects of hypoxia produced by a simulated high altitude, or lowered tension of oxygen on the natural resistance of the canary towards infections with *P. cathemerium*, as well as on the effect of an oxygen-deficient environment on the effectiveness of chemotherapy of the same infection.

MATERIALS AND METHODS

P. cathemerium (strain 3M)† was employed as the infecting agent in adult female canaries. The strain of avian malaria has been maintained in this laboratory since 1940 and routinely transferred by means of infected blood injected intramuscularly or intravenously into normal birds. We have seen no evidence of alteration in degree of parasitemia nor any change in response to therapy during this period of time.

The experiments reported herein involve the use of hypoxia which was obtained by use of a specially constructed steel chamber described by Kolls and Loevenhart.[2] The mechanism for regulation of constant pressure within the chamber has been modified by a simple automatic control device described by Loehning et al[3] by means of which any desired low pressure could be readily maintained. Access to the interior of the chamber was through an appropriate aperture which could be opened or closed as necessary. Food and water were supplied daily. To preserve the periodicity of *P. cathemerium*, the chamber was illuminated during the day, and an automatic control switch regularly turned this light off at 6:00 p.m. and on again at 6:00 a.m.

Blood films were made in the morning prior to

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† Obtained through the courtesy of Professor W. H. Taliaferro of The University of Chicago.

1. Gajewski, J. E. and Tatum, A. L. 1944, A study of the mechanism of relapse in avian malaria. J. Infect. Dis. **74**: 85–92.

2. Kolls, A. C. and Loevenhart, A. S. 1915, A respiratory chamber for small animals. Am. J. Physiol. **39**: 67–76.

3. Loehning, R. W., Zapata-Ortiz, V., Hughes, F. W. and Tatum, A. L. 1953, Effects of cocaine on rats subjected to low tensions of atmospheric oxygen. J. Pharmacol. & Exper. Therap. **108**: 80–86.

schizogony. These films were fixed in absolute methyl alcohol and stained by a slight modification of the Kingsley[4] technique. After fixation for approximately 5 minutes, the films were air-dried and then flooded with a mixture consisting of 10 drops of mixed "Kingsley stains" diluted with nine drops of distilled water.‡ They were stained for 14 minutes, washed for 3 or 4 minutes in a stream of distilled water, and then dried in air.

EXPERIMENTAL PROCEDURES AND RESULTS

It had been demonstrated in this laboratory that subjection of canaries to simulated altitudes or hypoxia has an adverse effect on the acquired "immunity" of this host to infections with *P. cathemerium*, as indicated by the fact that relapses of latent infections occur in all birds on the 3rd or 4th day at simulated altitudes of 18,000 ft or above. In the present studies noninfected canaries were placed in the low oxygen-tension chamber and the pressure reduced to a simulated altitude of 19,000 to 20,000 ft (approximately 75 mm Hg partial pressure of O_2). On the first time of introduction of a group of birds into the chamber the decompression was carried out at a slow rate, taking 18 to 22 hours to reach the desired "altitude," and with this procedure there were no fatalities. Subsequently this slow decompression was less necessary to the same group of birds. After maintaining these noninfected birds at this simulated high altitude for a period of 2 or 3 days, they were removed from the chamber and injected intravenously with an inoculum calculated to contain 5000 malarial parasites. They were then treated immediately by an intravenous injection of Primaquine and promptly returned to the low pressure chamber. Control birds at normal atmospheric pressure were checked in the same manner for the "curative" or prophylactic effect of several dosage levels of Primaquine. In table 1 are presented data indicating the "curative" or prophylactic effects of the different dosages of this drug under conditions of room atmosphere (860 ft) and that of atmospheres definitely and specifically deficient in oxygen (19,000 to 20,000 ft, simulated altitude). The period of 3 or 4 days in this hypoxic environment was adequate to produce a significantly diminished effectiveness of this drug.

The question then arose as to whether or not this period of 3 or 4 days was the optimal period of exposure to oxygen-deficiency in order to produce these maximally deleterious effects. Therefore, another group of birds was placed in the chamber and the pressure slowly reduced to a simulated altitude of 19,000 to 20,000 ft. After various intervals of time in this oxygen-deficient environment, they were removed momentarily, inoculated intravenously with 5000 parasites and immediately thereafter given 150 μg of Primaquine, also administered intravenously. After treatment they were promptly returned to the chamber. In table 2, the time of 0 hours at this simulated altitude means that the birds were injected as soon as feasible after the desired simulated altitude was attained. This time was approximately 18 hours—the time necessary to initially reach the desired "altitude" without fatalities.

The results appear to indicate that some slight adjustment to the hypoxic state commences shortly after exposures to the simulated high altitude but this acclimatization continues to increase only very slowly. By 16 days the birds' capacity to aid the drug in destruction of the parasites had returned to only

4. Kingsley, D. M. 1935, A new hematological stain. I. Constituents and methods of use. Stain Technol. 10: 127–133.

‡ Proportions determined by trial with the individual pipettes employed.

TABLE 1.—*Comparison of intravenous treatment immediately following intravenous inoculation of 5000 parasites* (P. cathemerium) *at ordinary room atmosphere, with similar inoculation and treatment after exposure of the birds to a simulated altitude of 19,000 to 20,000 ft for a period of 3 to 4 days.*

Dosage of Primaquine per bird	Room atmosphere			Exposure to simulated altitude of 19,000 to 20,000 ft		
	Number of birds	"Cured"	% "cure"	Number of birds	"Cured"	% "cure"
50 μg	4	2	50			
75 μg	4	3	75			
100 μg	5	4	80			
150 μg	21	21	100	23	6	26
300 μg	2	2	100	5	0	0
500 μg	1	1	100	6	1	16
1 mg				1*	0	0

* At this dosage level 9 of the 10 tested birds died from the acute toxic effects of the drug.

37.5% of that in normal air. A need of the chamber for other experiments prevented long-term adaptation experiments at this time. It is evident, however, that the return towards normality is in progress and that complete adaptation probably would require a much longer period than sixteen days.

The question then arose as to how quickly these effects of hypoxia are eliminated by returning the birds to the normal tension of oxygen in room air (altitude, 860 ft). There is a possibility that the adaptation just mentioned might be a recovery phase and that at 16 days the birds are only partially recovered from this adverse effect rather than partially adapted to it. Birds were exposed, then, to an atmospheric pressure corresponding to an altitude of 19,000 to 20,000 ft for 4 days. They were then removed from the chamber and separate groups of these birds were maintained for 1, 2 or 4 days in normal room air. After these intervals in normal room air each bird of a group was given 5000 parasites (*P. cathemerium*) intravenously and immediately treated, also intravenously, with 150 μg of Primaquine.

In table 3 it can be seen that 24 and 48 hours after removal from the chamber a 71% cure was obtained. This can be compared with approximately a 25% cure if they were treated and kept in the oxygen-deficient atmosphere and with a 100% cure which was obtained in birds not exposed to these conditions of low concentrations of oxygen. After an interval of 4 days in normal atmospheric conditions, there was a complete recovery, indicated by the 100% cure. This is a strikingly rapid recovery if it is compared with that of the birds listed in table 2, which were maintained at an

TABLE 2.—*Onset and duration of adverse effects on the host after various intervals of exposure to simulated altitudes of 19,000 to 20,000 ft, 5,000 parasites inoculated intravenously and immediate treatment with Primaquine (150 μg per bird).*

Time of exposure to 19,000 to 20,000 ft	Number of birds	Number cured	% cure
0 hours	9	1	11
24 hours	8	1	12.5
72 hours	12	3	25
96 hours	13	3	23
10 days	3	1	33
16 days	8	3	37.5

TABLE 3.—*Recovery of birds exposed to simulated altitudes of 19,000 to 20,000 ft for 4 days, then removed from the chamber to room atmosphere and given 5,000 parasites intravenously and 150 μg Primaquine intravenously after the lapse of 1, 2 and 4 days, respectively.*

Days after removal from chamber	Number of birds	Number cured	% cure
1 day	7	5	71
2 days	7	5	71
4 days	6	6	100

increased "altitude" level for much longer intervals with only slight adaptation.

In all of the above experiments control birds were inoculated intravenously with 5000 parasites but received no antimalarial therapy. This was used as a check to be certain that there was no alteration in the infectivity of this organism. If the number of parasites that is injected is known one can estimate approximately the day on which the organisms will appear in the blood stream in sufficient numbers to be accurately counted. In figure 1, typical courses of parasitemia are charted to illustrate the difference between that of non-treated room and hypoxic control birds. Bird number 963 is representative of the normal course of infection of a group of room control birds that received 5000 parasites intravenously. The parasites were first seen in a blood film on the 6th day after inoculation in a concentration sufficiently high to be accurately counted. Birds numbered 955 and 960 were exposed to a simulated altitude of 19,000 to 20,000 ft for 3 or 4 days and were then given the same number of parasites (5000) as were received by bird 963. They were maintained at this "altitude" for the duration of this experiment. Evidently the two-thirds destruction of parasites, which represents the natural immune response of the avian host,[5] did not occur in these hypoxic birds.

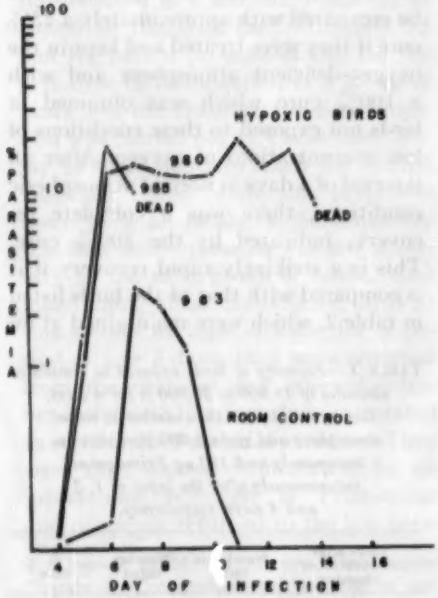

FIG. 1.—Typical course of hypoxic and of control birds infected with *P. cathemerium*.

Exposure to low atmospheric pressures has been stated to induce a hypersecretion and eventually an exhaustion of the adrenal cortex.[6] For this reason it was decided to determine whether or not comparable results could be obtained by using cortisone as a means of inhibiting the defense mechanisms of this avian host. Previously unreported work performed in this laboratory had demonstrated that 100 mg of cortisone per kilogram body weight administered daily to canaries subcutaneously or intraperitoneally for 7 to 10 days is an adequate dosage to cause a relapse of all of the tested canaries with latent infections. Since these birds consistently relapsed with cortisone therapy, the question arose as to whether or not cortisone administered previously to treatment could have any influence on antimalarial therapy. Canaries were given 100 mg/kg cortisone (Cortone, Merck) subcutaneously daily for 10 days. After this treatment 5000 plasmodia were administered intravenously, followed by an immediate intravenous administra-

5. Boyd, G. G. 1939, A study of the rate of reproduction in the avian malaria parasite, *Plasmodium cathemerium*. Am. J. Hyg. Sec. C. **29**: 119–129.

6. Goldzieher, M. A. 1950, The role of the adrenal gland in the utilization of oxygen. J. Aviation Med. **21**: 153–159.

tion of 150 μg of Primaquine. Ten birds were tested and six were "cured" at this dosage level. This amount of Primaquine administered in the same manner "cured" 100% of the control non-cortisonized canaries.

The course of infection was also charted in a group of cortisonized, infected, but otherwise untreated birds. This was compared to untreated birds which received no cortisone but which received the same inoculum of parasites. Figure 2 represents the typical course of the parasitemia of birds after having received 100 mg/kg of cortisone subcutaneously daily for a period of 10 days. They then received an injection intravenously of 5000 parasites, and cortisone treatment was continued until the termination of the experiment.

The course of infection in cortisonized birds is quite similar to that seen in birds subjected to hypoxia. The upswing of infection is also more rapid

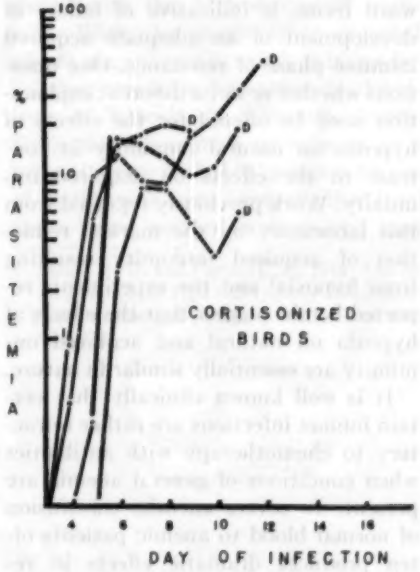

FIG. 2.—Typical course of infection of cortisonized birds.

than in nontreated room birds, a higher peak is reached, and there is a consistent failure of the birds to start the downward recovery phase or curve of the infection. There is a sustained climactic peak of parasitemia until the birds expired on the 9th to the 12th day after being inoculated.

DISCUSSION

While some evidence has been reported to the effect that a protective antibody is present in the blood of avian and other hosts during and after malarial infections, the increased functional capacity of the reticulo-endothelial system has been generally considered to be the chief mechanism for keeping the blood of the host relatively free from parasites during the state of latency. It can be postulated, then, that an oxygen-deficient environment has an adverse effect on birds infected by *P. cathemerium*, through a depression or stasis of the proliferative or functional mechanism of the fixed phagocytic cells of the host. This is postulated with the knowledge that a factor of primary importance in the resistance of the canary is the generation of new macrophages with increased phagocytic activity. It is to be generally considered that in most infections the response of the host is a necessary contributing factor in the destruction of the invading organisms. This concept is strongly supported by the fact that the effective in vitro concentration of Primaquine and other chemotherapeutic agents far surpasses the calculated dosage to be administered intravenously in order to destroy the same number of parasites in vivo.

The increase in dosage of Primaquine required to "cure" birds subjected to hypoxia occurred almost immediately. The rapid onset of this unfavorable condition may be due in part to the initial

stress of decompression and not to a specific result of oxygen-deficiency, but the net effect is that a higher concentration of the drug is necessary, evidently because some deleterious effect has been induced in the host. The possibility that the drug may be altered by the host which is in this oxygen deficient environment appears to be remote. That the virulence of the infectious agent may have been increased appears unlikely, because inoculation of blood from either an hypoxic or a room control infected bird into recipient birds produced a parasitemic curve of infection which depended solely upon the condition of the recipient bird and the number of parasites that was injected.

It is interesting to note that an adaptation to an hypoxic environment begins immediately but progresses rather slowly thereafter. One may consider that this is as accurate a method of studying adaptation to various altitudes, simulated or otherwise, as a study of the various cellular elements of the blood and it appears that the effects of hypoxia in these instances are readily measurable. The delay of return to normal capacity of the birds might suggest that the adaptation to hypoxia is essentially a recovery reaction to the initial stress of decompression to these simulated altitudes. However, the acute effects of hypoxia were promptly removed by placing these birds in normal air in contrast to the long period of time required for adaptation to continued hypoxia.

A study of the courses of parasitemia of hypoxic canaries in contrast to birds in normal air leads one to compare the effects on both natural and acquired immunity. Since a normal destruction of two thirds of the parasites usually occurs in a normally susceptible bird, this destruction has been considered to constitute a measure of natural immunity. In a bird, in a state of hypoxia, this destruction of two thirds of the parasites does not occur. The parasites appear earlier in the blood stream, and the rate of increase of the level of parasitemia is such as to indicate a failure of destruction of these organisms at the usual rate. This must then be considered to be a decrease in the natural immune forces of this host..

The normal course of parasitemia, in a nontreated bird in room atmosphere (altitude 860 ft), shows that after the critical peak of the infection occurs, there is a rapid decrease of parasites until a state is reached in which the parasites are no longer to be seen in the blood. This fall in parasitemia has been generally accepted as an indication that the phagocytic system has risen to full capacity and that for a time at least, the parasites are destroyed at a greater rate than that at which they are produced. On the other hand, the sustained high peak of infection, with no downward trend, is indicative of failure of development of an adequate acquired immune phase of resistance. One questions whether or not a different explanation need be offered for the effects of hypoxia on natural immunity in contrast to the effects on acquired immunity. Work previously reported from this laboratory on the marked reduction of acquired immunity resulting from hypoxia[1] and the experiments reported herein suggest that the effects of hypoxia on natural and acquired immunity are essentially similar in nature.

It is well known clinically that certain human infections are rather refractory to chemotherapy with antibiotics when conditions of general anemia are present. In severe anemia, transfusion of normal blood to anemic patients often produces dramatic effects in response to administration of antibiotics. It may not be illogical to assume that

the relative ineffectiveness of the chemotherapy may be due, at least in part, to the harmful effects of generalized hypoxia analogous to the relative inefficiency of antimalarials in the conditions described above.

The similarity of the courses of the infections in animals treated with cortisone and in those subjected to hypoxia is quite marked and one might be tempted to explain the effects of hypoxia entirely on the hypothetical release of cortisone. However, the necessity of use of such high concentrations of cortisone to block the effectiveness of Primaquine suggests that this is not likely to be the entire explanation. The dosage of cortisone necessary (100 mg/kg) to cause a decrease in the effectiveness of Primaquine is higher than that which has been reported as the physiological concentration even under conditions of extreme stress.[7] Furthermore, the activity of Primaquine was decreased only to 60% of normal efficiency with this high dosage of cortisone. With the many other reported effects of cortisone in mind, it is our belief that cortisone does simulate the effects of hypoxia to a certain degree. Subjection of animals to hypoxia as a type of stress probably causes a release of cortisone in some form or other from the adrenal cortical sources and may produce other effects which may or may not be associated with the concentration of the circulating cortisone.

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SUMMARY

The minimal "curative" (or suppressive) dosage of Primaquine for canaries infected by *Plasmodium cathemerium* in normal air, at slightly over 800 ft altitude, must be increased approximately tenfold for similarly infected canaries which have been maintained in an atmosphere deficient in oxygen (75 mm Hg partial pressure) for a period of 3 or 4 days. In other words, hypoxia markedly reduces the effectiveness of antimalarial agents.

The marked decrease in effectiveness of the drug in conditions of hypoxia occurs quickly and adaptation to this environment is very slow (over 16 days). However, removal of birds from the oxygen-deficient environment to normal air causes a very prompt recovery from the harmful effects of hypoxia.

The course of infection with *P. cathemerium* in canaries which have been subjected to simulated altitudes of 19,000 to 20,000 ft bears a considerable similarity to the course of infection of birds receiving daily injections of cortisone (100 mg/kg).

Administration of cortisone, previous to infection, reduces the effectivity of Primaquine but this effect is not as marked as that obtained with the reduction of the tension of oxygen as employed in these experiments.

THE SEROLOGIC CHARACTER OF CHOLERA VIBRIO MUCINASE

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Burnet and Stone (1947a) described an activity present in cultures of *Vibrio cholerae* that produced a desquamation of the intestinal mucosa of the guinea pig. They regarded this activity as enzymatic in nature and showed that it was distinct from the receptor destroying enzyme (RDE) also produced by the cholera vibrio. The authors referred to this new enzyme as mucinase. They found that vibrio mucinase could be specifically inhibited by antiserums and that absorption with living vibrios did not decrease the neutralizing power of these antiserums, even though 98% of agglutinating antibodies were absorbed.

Similar results were reported by Singer, Wei and Hoa (1948), who showed that mucinase activity was present in culture filtrates but not in washed suspensions or autolysates of the vibrios. They also found, however, that the activity was neutralized not only by antiserums prepared from culture filtrates but also by antiserums to the 0 antigen of vibrios and that both types of antiserums lost their neutralizing power after absorption with boiled vibrio suspensions.

Using a different method of demonstrating vibrio mucinase, Burnet (1948) showed that it was active against various glandular mucins but not against synovial fluid mucin. He (Burnet, 1949) later reported that ovomucin could be used as a substrate for vibrio mucinase and described a method of titration based on depolymerization of ovomucin such that it was no longer precipitated by protamine sulfate. Using this latter method, Jensen (1953) further studied the mucinase activity and its inhibition by antiserum. He showed that antiserums to culture filtrates have a high neutralizing power, while 0 antiserums are ineffective, thus confirming Burnet's (1947) earlier results.

Narayanau, Devi and Menon (1953) and Gurkipal Singh and Ahuja (1953) confirmed the presence of mucolytic activity in cultures of cholera vibrios. They found that some El Tor and non-agglutinating water vibrio strains also produced enzymes capable of desquamating intestinal epithelium and of reducing the viscosity in ovomucin solutions.

Lam and Mandle (1954) demonstrated that vibrio mucinase increases the permeability of isolated intestinal loops from the mouse. They were able to neutralize this effect by active and passive immunization against the homologous mucinase.

While the antigenicity of vibrio mucinase, as distinct from the 0 antigen, may be regarded as tentatively established, there is as yet little evidence as to its serological homogeneity. Singer, Wei and Hoa (1948) and Jensen (1953) used only a single vibrio strain in their studies. Burnet and Stone (1947a) suspected a third enzyme which might further break down the cement substance between cell walls of the guinea pig ileum. They did not, however, mention any serologic differences among the mucinases from seven vibrio strains and three antiserums used in their studies. Burnet (1949) mentions a third enzyme which does not act on intact ovomucin. Referring to the ovomucin

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test, he states that "nothing has been observed to suggest, that more than two enzymes [RDE and mucinase] and their corresponding antibodies are concerned."

It has been demonstrated earlier (Freter, 1951), however, that the hemolysins and probably the gelatine liquefying enzymes of water and cholera vibrios show marked serological differences in inhibition tests with antiserums. As an essential preliminary to investigation of the relation, if any, of the mucolytic activity to the pathogenicity of *V. cholerae*, the serological character of mucinase activity of a group of strains of the cholera vibrio from widely different sources has therefore been investigated. Narayanau, Devi and Menon (1953) and Gurkipal Singh and Ahuja (1953), who had found similar mucolytic activities in cholera as well as certain nonpathogenic vibrios, did not attempt to differentiate these enzymes serologically. In order to investigate this point, a number of water vibrios isolated in Chicago have also been tested. The results of these studies are reported here.

MATERIALS AND METHODS

Vibrio strains.—The experiments were carried out on mucinase prepared from cultures of: (1) Nine vibrios isolated in 1953 from cases of cholera in Calcutta: strains C41, C144, C171–175, C401 and C441, all of the Ogawa type, except C401, which agglutinated with specific Inaba serum. Strains C144, C401 and C441 were isolated from different fatal cases, the rest of the strains from patients who recovered later. The series C171–C175 represent successive daily isolates from one patient. (2) Four vibrios isolated earlier in India: NIH 41 c2 (Ogawa), Bengal T 12 V (Ogawa), Bengal F 29 V and Bengal F 34 V (Inaba). (3) Three vibrios isolated in 1947 in Egypt: Egypt 18 and Egypt 495, both isolated from cholera patients, and Egypt 5749, isolated from a carrier (types not determined). (4) One strain from Indochina: Dalat 85, isolated in September 1951 (type not determined). (5) Three water vibrios isolated in 1953 in the vicinity of Chicago: CD, JP, and LC. These strains did not agglutinate with 0 antiserum to *V. cholerae*.

As has been reported elsewhere (Husain, 1955), three different types of vibrios can be distinguished with respect to their colonial morphology on thionin agar plates: type A, growing in translucent colonies, type B, growing in dense colonies and type C, growing in colonies with concentric striation. It has been found (Burrows, 1953) that, of these colonial types, only that showing concentric striations occurs on primary isolation plates of thionin-glycerol agar; it may be assumed, therefore, that this represents the typical colonial morphology of the "true cholera vibrio." All Calcutta strains used in the present study belonged to this striated colonial type (C). The other cholera vibrios are not as homogeneous, possibly due to the longer cultivation on artificial mediums:

Type A: strains Bengal T 12 V and Dalat 85.

Type B: Strains F 29 V, Egypt 18 and Egypt 495.

Type C: Strains NIH 41 c2 and Egypt 5749.

Mucinase.—The vibrios were grown in thionin glycerol agar (Husain, 1955) at 37 C for 14 to 16 hours. The agar was then removed, mixed with distilled water and emulsified in a blendor. After centrifugation, the clear supernate was lyophilized. The reconstituted material was used for the titration of mucinase activity and for the immunization of rabbits. It must be realized, of course, that in all probability these preparations contain besides cell substances and metabolic products a number of other emzymes as well as mucinase. The only specificity of the test described below is therefore introduced by the substrate.

Anti-mucinase serums.—Antiserums were prepared from rabbits by intravenous injection of increasing amounts of mucinase preparation (0.5 to 2 ml) in 5-day intervals. The animals were bled 8 days after the last injection. Four to six injections were usually sufficient.

Standard ovomucin.—Ovomucin was prepared from egg white by a simplification of the method of Young (1937). The whites of 12 eggs were freed of chalazae and poured into 4 liters of distilled water at 0 C. The precipitate formed after 30 minutes of stirring was centrifuged off, dissolved in 5% NaCl solution and finally diluted with distilled water to a salt concentration of 0.85%. Undissolved material was removed by centrifugation. The supernate was diluted with 0.85% NaCl to a concentration such that a further dilution of 1:2 gave a precipitate with protamine sulfate, but that a 1:4 dilution did not. All data given below in any one table are derived from titrations with the same batch of ovomucin.

Titration.—Mucinase preparations in 0.25 ml amounts were diluted 2^n in 5% normal or im-

mune serum in Wassermann tubes in an ice bath. After incubating at 37 C for 10 minutes the tubes were transferred back into the ice bath, an equal amount of ovomucin was added and the incubation continued for 1 hour at 37 C. All reagents contained 0.01% merthiolate. After chilling the tubes, undigested ovomucin was detected by precipitation with protamine sulfate (1 drop of 0.1% solution). The highest dilution in which protamine sulfate gave no precipitate was taken as the mucinase titer. This technique of mixing different antigen dilutions with constant amounts of antibody was employed rather than the reverse procedure in order to make the results comparable to previous investigations (Burnet, 1949; Jensen, 1953), even though Jensen (1953) has shown that both methods of titration gave identical end points with his mucinase-antimucinase system (unlike the precipitin system). Another reason for selecting the antigen dilution method was that it dispensed with preliminary enzyme titrations to obtain antigen of standard activity.

RESULTS

Table 1 presents the results of cross neutralization tests with strains recently isolated in India. The fractions in this and the other tables represent the value

$$\frac{\text{mucinase titer in 5\% normal serum}}{\text{mucinase titer in 5\% antiserum}}$$

termed "fractional inhibition." The "fractional inhibition" of 1/16, for example, recorded in table 1 (and 2) for a given antiserum and mucinase, thus simply indicates that this antiserum depressed the titer of the particular mucinase to 1/16 of its titer in 5% normal serum.

The unit of mucinase is defined as the minimal amount of enzyme which in the described test is capable of rendering 0.25 ml of standard ovomucin not precipitable with protamine sulfate. The antimucinase content of a given serum is consequently expressed as the number of mucinase units neutralized by the amounts of serum used in the test (0.25 ml of a 5% dilution). If, for example, the mucinase of strain C175 gives a titer of 1:512 in normal serum and of 1:8 in antiserum C175, the fractional inhibition shown in the tables is

$$\frac{1:512}{1:8} = \frac{1}{64}.$$

Thus one mucinase unit was present in the 1:512 dilution of mucinase C175 in normal serum, and therefore 64 units were present in the 1:8 dilution. On this basis the antimucinase content of antiserum C175 was taken to be 64 units (table 1). The number of antimucinase units in a given antiserum is, therefore, equal to the reciprocal of its "fractional inhibition" value.

It can be seen from table 1 that not all serums inhibited enzymes from heterologous strains to the same degree as the homologous mucinases. This result suggested that more than one type of mucolytic enzyme might be present in cholera vibrios. In order to determine the degree of this heterogeneity, twelve more mucinases from different vibrio strains were tested together with three

TABLE 1.—*Neutralization of mucinase preparations by different antiserums.*

Mucinase from strain no.	Mucinase titers in normal serum	Fractional inhibition = $\frac{\text{mucinase titer in 5\% normal serum}}{\text{mucinase titer in 5\% antiserum}}$ by antiserums:							
		C171	C172	C173	C174	C175	C144	C401	C441
C171	1:256	1/64	1/4	1/32	1/32	1/64	1/128	1/32	1/128
C172	1:1024	1/8	1/16	1/128	1/8	1/4	1/128	1/32	1/512
C173	1:512	1/16	1/16	1/64	1/4	1/4	1/128	1/64	1/256
C174	1:512	1/64	1/4	1/16	1/32	1/64	1/128	1/32	1/128
C175	1:512	1/128	1/4	1/32	1/32	1/64	1/128	1/32	1/128
C144	1:1024	1/8	1/8	1/64	1/4	1/2	1/128	1/128	1/256
C401	1:512	1/16	1/16	1/32	1/4	1/4	1/64	1/64	1/128
C441	1:2048	1/8	1/16	1/64	1/8	1/4	1/256	1/32	1/256
Antimucinase units in serums when tested with homologous mucinase		64	16	64	32	64	128	64	256

enzymes of the above group (table 2).

The data in table 2 seem at first to indicate a large number of serological types but it appears more probable that only two types occurred in the strains studied. The variability in cross neutralization titers may then be explained on the assumption of different mixtures of both types present in the tested preparations. The quantitative distribution of these two mucinase types has been arrived at as follows:

The serological type of mucinase produced by strain C175 was arbitrarily designated type A. As can be seen from table 2, the mucinase produced by strain E5749 is serologically unrelated to that of C175 and has been designated type B. The slight cross reaction of C175 mucinase with E5749 antiserum is within the experimental error (± 1 dilution) but may suggest that a small amount of A-mucinase is produced by vibrio E5749.

Similar considerations allow the other enzymes and antiserums to be described quantitatively: mucinase C144, for example, is not inhibited by either antimucinase type A or B alone (as represented in antiserums C175 and E5749, table 2). But because antiserum C144 inhibits both mucinase types A and B (C175 and E5749) to approximately the same degree as the homologous enzyme, the latter must consist of both mucinase types in equal concentration (expressed A, B in table 2).

Mucinase C41 may be taken as another example. Antiserum C144 (containing antimucinase against both types A and B) inhibited this enzyme as strongly as the homologous mucinase. Of the 64 antimucinase A units of serum C175, however, only 16 were effective when tested with mucinase C41 (fractional inhibition = 1/16, table 2). Table 3 illustrates the assumed distribution of type A and B mucinases through the different dilutions of mucinase C41 in this reaction. The highest enzyme dilution which contains one or more units of mucinase (types A and B combined) is 1:512 (tube #9). This dilution is by definition the titer of C41 mucinase in normal serum because it is the highest dilution in which the ovomucin will not precipitate with protamine sulfate. If the type A mucinase is inhibited by antiserum C175, however, then the last tube containing one unit mucinase (type B) is tube #5 and consequently 1:32 is the mucinase titer of C41 in antiserum C175. The fractional inhibition for this reaction (as shown in table 2) is

$$\frac{1:512}{1:32} = \frac{1}{16}$$

The C41 mucinase may therefore be regarded as a mixture of types A and B, the amount of type A being 16 times greater than that of type B (expressed 16A, B in table 2).

Again taking mucinase C41 as an example, the assumption that it consists of a mixture of two serological types of mucinase has been further tested in the following way: If antiserum C175 contains only antibodies against type A mucinase, the 16-fold inhibition of C41 mucinase by this antiserum should be due only to the reaction with the A component, leaving the B component intact. An increase in the concentration of serum C175 should, therefore, not increase the degree of inhibition of mucinase C41 beyond the fractional inhibition of 1/16, because in the lower dilutions of C41 mucinase the B component should be sufficient to act on the ovomucin (table 3).

The results of this type of experiment are shown in table 4. Antiserum C175 did not inhibit mucinase C41 beyond the postulated degree. The inhibition of

TABLE 2.—*Neutralization of mucinase preparations by different antiserums.*

Mucinase from strain no.	Units and types of mucinase†	Mucinase titers in normal serum	Fractional inhibition = mucinase titer in 5% normal serum / mucinase titer in 5% antiserum by antiserums:														
			41 c 2	E18	E495	E5749	B T 12 V	B F 29 V	B F 34 V	D85	C41	C144	C172	C175	CD	JP
41 c 2	64A, B	1:4096	1/256*	1/64	1/64	1/2	1/256	1/16	1/32	1/64	1/64	1/256	1/16*	1/128	1/512	1/4
E18	4A, B	1:256	1/16	1/32	1/16	1/8*	1/64*	1/2	1/8	1/16	1/16	1/128	1/2	1/4	1/32	1
E495	128A, B	1:1024	1/64	1/32	1/32	1/4	1/256	1/4	1/8	1/64	1/16	1/256	1/2	1/128	1/64	1/2
E5749	B, little A	1:1024	1/8	1/32	1/4	1/16	1/4	1	1/16	1/2	1/16	1/64	1/4	1	1/16	1
B T 12 V	A, little B	1:1024	1/64	1/64	1/32	1/2	1/128	1/4	1/32	1/64	1/64	1/256	1/2	1/128	1/64	1
B F 29 W	32A, B	1:512	1/16*	1/16	1/16	1/4	1/64	1/8	1/32	1/16	1/16	1/64	1/4	1/32	1/64	1
B F 34 V	2A, B	1:1024	1/8	1/16	1/4	1/4	1/8	1/4	1/16	1/8	1/32	1/128	1/8	1/4	1/32	1/2
D85	A, little B	1:1024	1/64	1/64	1/64	1/4	1/256	1/8	1/8	1/32	1/8*	1/256	1*	1/64	1/64	1
C41	16A, B	1:512	1/32	1/32	1/32	1/4	1/64	1/4	1/8	1/32	1/32	1/128	1/8	1/16	1/128	1/4
C144	A, B	1:512	1/2	1/32	1/4	1/2	1/4	1	1/4	1/4	1/32	1/64	1/8	1/2	1/64	1/2
C172	A, B	1:2048	1/8	1/32	1/2	1/2	1/8	1	1/8	1/8	1/16	1/64	1/8	1/2	1/16	1
C175	A	1:1024	1/64	1/64	1/32	1/2	1/64	1/8	1/8	1/64	1/16	1/256	1/4	1/64	1/256	1/2
CD	?A?	1:1024	1/16	1/128	1/16	1/8	1/16	1/2	1/16	1/64	1/64	1/256	1/2	1/128	1/512	1
JP	?B?	1:512	1/8	1/128	1/4	1/4	1/8	1	1/8	1/8	1/32	1/32	1/4	1/2	1/128	1/32
LC	?	1:512	1/2	1/2	1/2	1	1/4	1	1	1/4	1/2	1/8	1	1	1/32	1/4
Units and types of antimucinases†			64A 4B	32A 32B	32A 4B	2A 16B	128A 4B	8A	16A 8B	32A 4B	32A 16B	128A 64B	4A 4B	64A	?	?

* Error ± 2 dilutions.
† Explanation in text.

TABLE 3.—*Units of type A and B mucinases assumed in the different dilutions of mucinase C41.*

Tube no.	1	2	3	4	5	6	7	8	9	10
Dilution of mucinase C41	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024
Units of mucinase A assumed in tubes	256	128	64	32	16	8	4	2	1	1/2
Units of mucinase B assumed in tubes	16	8	4	2	1	1/2	1/4	1/8	1/16	1/32

homologous mucinase, however, is increased with higher concentrations of serum C175.

Following the reasoning outlined in the preceding paragraphs, the mucinases prepared from cholera vibrios (excluding those from the water strains CD, JP and LC) could be described as consisting of two serological types, A and B (table 2). These descriptions could be made consistent with 137 of the 144 titrations recorded in table 2 if an experimental error of ± 1 dilution was permitted. In 7 of the 144 titrations (marked with an asterisk) an experimental error of ± 2 dilutions had to be assumed in order to fit the results into the suggested pattern. This leaves over 95% of the data within the customarily accepted margin of accuracy for serological titrations (± 1 dilution). The assumption that the tested mucinases and antimucinases are mixtures of only two serological types is therefore consistent with the experimental results within statistically acceptable limits ($P = 0.05$). Additional serological types of mucinase appear to be formed by the water vibrio strains, even though the enzymes of two of them (CD and JP) are closely related to the mucinases from cholera vibrios.

The above conclusions regarding the distribution of mucinase types A and B among cholera vibrios are, of course, based on the assumption that there is a regular relationship between antigen and antibody in the reaction. It has been reported by Jensen (1953) that this relationship is both direct and linear. This observation could be confirmed, but in some cases a more complicated relationship was found (see table 4, inhibition of mucinase C175 with different concentrations of homologous antiserum). The quantitative distribution of mucinase types A and B among the cholera strains, as indicated in table 2, must, therefore, be considered as only an approximation in some cases, a fact which might explain the 5% incidence of larger experimental errors (± 2 dilutions). Even in the case of mucinase-antimucinase C175 (table 4), however, the "fractional inhibition" value of the antiserum is a fairly good indicator of antibody concentration (unlike the end point in precipitin titrations with constant amounts of antiserum, which has little relation to antibody concentration).

The demonstrated existence of only two types of mucinase in strains of *V. cholerae* might raise the question whether one of these components could be identical with the receptor destroying enzyme, RDE, which according to Burnet (1949) is antigenically different from mucinase but has a similar action on ovomucin. This possibility must be excluded, however, from the properties of RDE as described in the literature:

TABLE 4.—*Effect of antiserum concentration on titers of different mucinases.*

Mucinase dilutions made in:	Mucinase C175		Mucinase C41	
	Titer	Fractional inhibition	Titer	Fractional inhibition
20% normal serum	1:1024		1:512	
20% serum C175	1:4	1/256	1:64	1/8
5% serum C175*	1:32	1/32	1:32	1/16
1.25% serum C175*	1:256	1/4	1:256	1/2

* Normal serum added to make a final serum concentration of 20%.

(1) RDE is not antigenic if given intravenously to rabbits (Burnet and Stone, 1947b; Burnet, 1949). Both mucinase types described in the present paper were antigenic by the intravenous route.

(2) RDE is spontaneously inactivated in cultures at high pH (Burnet, 1948; Stone, 1949; Nihoul, 1951). The mucinases used in the reported experiments were prepared from thionin agar cultures at pH 8.2 to 8.5.

(3) RDE acts only slowly on ovomucin. Three hours of incubation at 37 C are necessary and little effect can be seen after 1 hour (Burnet, 1949). In all titrations described here incubation has been for 1 hour only at 37 C.

(4) RDE renders ovomucin nonprecipitable with protamine sulfate but leaves it still precipitable with cetyl trimethyl ammonium bromide (CTAB) while ovomucin acted upon by mucinase does not precipitate with either one of these reagents (Burnet, 1949). Both mucinase types A and B, as represented by mucinases C175 and E5749, respectively, did not show any difference in titer when tested with protamine sulfate or CTAB.

(5) Mucinase C144 which, as the above data indicate, contains both mucinase types A and B, did not cause any T agglutination of chicken cells in a test used for the titration of RDE (Burnet and Stone, 1947b).

DISCUSSION AND CONCLUSIONS

Burnet and Stone (1947a) and Lam and Mandle (1954) demonstrated that the mucolytic enzyme of *V. cholerae* is capable of desquamating intestinal mucosa and of increaing the permeability of isolated intestinal loops. These observations raise the question of whether or not any conclusions as to the virulence of a given vibrio strain can be drawn from either the activity or from the serological type of mucinase produced. From the present studies this has to be denied on the following grounds:

(1) The most striking agrument is the occurrence of high titer mucinases, serologically related to enzymes from cholera vibrios, in nonagglutinating and presumably avirulent water strains from Chicago. (2) No consistent difference in titer or serological composition of mucinases could be found when recently isolated strains from fatal cholera cases (C144, C401, C441) were compared with strains isolated from patients who recovered later (C41, C171–175, see tables 1 and 2). (3) Mucinase titers may differ considerably in different batches prepared from the same strain. (4) A given vibrio strain may produce different titer mucinase in different media, even though the rate of growth is comparable. Therefore, mucinase titers of in vitro cultures do not justify any conclusions as to the enzymatic activities of a given vibrio strain in vivo unless the in vivo environment could be sufficiently simulated in artificial mediums, which, at present, is not possible.

It may be emphasized in this connection that only one batch of mucinase has been prepared from most of the tested strains. No attempts have been made to determine the variability of vibrio strains with respect to their production of type A and B mucinases. The markedly different composition of mucinases from successive isolates of one patient (C171 and C175) might certainly suggest such a variability, even though other mechanisms like selection of mutants in the patient, for example, can also be suspected.

The data presented indicate further that there is no relation between colonial morphology and mucinase type produced. The Calcutta strains, for example, which all grow in colonies of the same type (C), exhibit different

combinations of mucinase types in their culture filtrates. The same must be said for the relation between structure of O antigen and the serological type of mucinase produced, considering that all Calcutta strains (except C401) belong to the Ogawa type.

In general, then, it can be concluded that neither the virulence, colonial morphology or O antigen structure of cholera vibrios has any relation to the titer and serological type of mucinase produced in vitro and tested with the above method. The data presented do, of course, not give information as to the actual relation of mucinase or other enzymes to the pathogenciity of cholera vibrios. However, the serological heterogeneity of cholera vibrio mucinases, demonstrated in this paper, must be considered in further investigations concerned with this problem.

SUMMARY

Cross neutralization tests in all possible combinations of a group of 20 representative vibrio mucinases and their homologous antiserums have been carried out. The results are consistent with the assumption of two serological types of mucinase, occurring in varying combinations in the several preparations.

The characteristics of both type of mucinase were distinct from those reported for RDE.

Three strains of nonagglutinating water vibrios isolated in Chicago were shown to produce high titer mucinases, which in two cases were serologically related to the enzymes of cholera vibrios.

The data presented indicate that the titer and serological type of mucinase produced in vitro has no relation to virulence, colonial morphology or O antigenic structure of the tested cholera strains.

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TOXIC END PRODUCTS FROM *PASTEURELLA PESTIS*

II. TOXIN YIELDS AS INFLUENCED BY CONDITIONS OF GROWTH

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In a previous study[1] it was demonstrated that bile salts brought about rapid dissolution of the cells of *Pasteurella pestis* with the release of end products toxic for mice. These toxic end products, as well as those resulting from bile salt action on cell-free lysates, were relatively labile and possessed other properties which suggested the possibility of a new entity. In the course of these experiments it became apparent that yields of toxin varied with seemingly minor differences in conditions of growth. The present report concerns the potentialities as to yields of toxin as related to environment during the growth and lysis of the bacterial cells.

METHODS AND MATERIALS

The methods employed have been described in detail in the previous report.[1] The culture used was *Past. pestis*, E.V. 76, "avirulent." The conditions of growth are presented with each experiment.

The term "lysate toxin" refers to that derived by the method of Jawetz and Meyer.[2] Suspensions of agar grown organisms are held at 37 C for 48 hours and then at 4 C for 24 hours. The cell-free supernate is the "toxin." The term "desoxycholate toxin" refers to either of two preparations as previously described, viz., the supernate obtained from agar grown suspensions treated with sodium desoxycholate to a final concentration of 0.5%, or to a cell-free lysate similarly treated with the bile salt.

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1. Goodner, K., Pannell, L., Bartell, P. and Rothstein, E. L. 1955, Toxic end products from *Pasteurella pestis*. I. A comparison of lysate toxin with that obtained from the action of bile salts. J. Infect. Dis. **96**: 82–87.
2. Jawetz, E. and Meyer, K. F. 1943, Avirulent strains of *Pasteurella pestis*. J. Infect. Dis. **73**: 124–143.

EXPERIMENTAL

Effect of growth temperature upon the toxicity of cell suspensions.—Favarel[3] suggested that living avirulent *Past. pestis* used in immunization was less likely to give severe local and general reactions in man if grown at 26 C rather than at 37 C. In order to confirm this observation the toxicity of "avirulent" organisms grown at various temperatures was determined. Roux bottles containing blood agar base were seeded from a broth culture and distributed to incubators maintained at various temperatures. After 3 days, the growths were harvested in physiologic saline. Plates were inoculated with aliquots of suitable dilutions for estimation of viable populations. The suspensions were then diluted in a fourfold series and mice injected intraperitoneally. The animals were observed for 10 days.

From the data as to viable counts and with reference to harvest volume and agar surface, estimates were prepared as to comparative populations at various growth temperatures. From the LD_{50} results with the various suspensions, calculations were made so as to give the number of LD_{50}'s per 1×10^9 viable cells with each suspension. These results are shown in table 1.

It is evident that the higher temperatures were distinctly unfavorable to the vegetative multiplication of *Past. pestis*. On the other hand, cells grown at

3. Favarel, R. 1949, Peste: Arch. de l'Inst. Pasteur ce Tananarive, 9–17.

TABLE 1.—*Toxicity of* Past. pestis (*E.V. 76*), *avirulent with reference to temperature of growth.*

Temperature of growth	Cell crop: viable organisms /cm² agar surface	Lethal quality of cells $LD_{50}/10^9$ viable cells
26 C	4.4×10^9	23
32	2.0×10^9	18
37	6.7×10^7	71
39	5.8×10^6	559
42	6.7×10^6	1370

the higher temperatures proved to be either more toxic or more infectious. The distinction cannot be absolute since some multiplication has been shown to occur in vivo.[4] The sharp break in the magnitude of cell crop occurred at about 37 C. This, too, was the approximate break in the curve of lethality. It is of interest that the organisms grown at 42 C appeared to be 62 times as lethal, cell for cell, as those grown at 26 C, the optimum for vegetative multiplication.

The relation of growth temperature to toxin yields.—The foregoing observation as to toxicity of suspensions with reference to growth temperature suggested that the potentiality of toxin yields might follow the same pattern. This point is of practical interest when considering the fact of fever temperatures in the infected animal.

To test hypothesis a long series of experiments was carried out on two varieties of toxin yields: (a) lysate toxin, (b) desoxycholate toxin. Although in each individual experiment the results were consistent there was great variation in toxin yields from one experiment to another due to factors not understood. The variation in toxin yields with temperature of growth and the divergence of results within given temperature ranges have been summarized in table 2.

In general, the yields of toxin, whether lysate or desoxycholate type, were greater the higher the incubation temperature if considered with reference to the number of viable cells. This holds irrespective of whether the extremes or the mean values are considered. The mean values with desoxycholate toxin approximate the lethality levels with whole cells.

Other experiments indicated that the temperature of incubation during the latter part of the growth cycle might be the decisive factor. It has been possible to demonstrate the temperature effect by initiating growth (48 hours) at 30 C, and then transferring the cultures to 41 C for 24 hours. This results not only in a significantly better cell crop but also in cells possessing high toxin potential. It should be pointed out, however, that *Past. pestis* grows quite slowly on agar and that the larger part of the growth is at 40 to 72 hours.

Influence of temperature of dissolution.—Since the temperature of growth appeared to be important for the development of toxin precursors it was of interest to determine the effect which temperature might have upon the release of toxins and/or precursors. Portions of one harvest (30 C grown) were allowed to stand at various temperatures for 48

TABLE 2.—*Influence of growth temperature upon the toxin potentialities of* Past. pestis.

Type of toxin preparation	Growth temperature	Examples included	Toxin: Units/10^9 bacteria	
			Range	Mean
Lysate	22–30 C	9	0.2– 3.0	1.1
	31–36	6	1.2– 4.1	2.1
	37–38	9	1 – 26	6.1
	39–40	5	4 – 39	17.8
	41–42	3	21 – 134	74
Desoxycholate	22–30	18	2 – 29.2	9.8
	31–36	5	4.4– 27	13.7
	37–38	17	3.1– 184	35.5
	39–40	5	27 –1040	384
	41–42	3	213 –3400	1434

4. Walker, D. L., Foster, L. E., Chen, T. E., Larson, A. and Meyer, K. F. 1953, Studies on immunization against plague. V. Multiplication and persistence of virulent and avirulent *Pasteurella pestis* in mice and guinea pigs. J. Immunol. **70**: 245–252.

TABLE 3.—*Temperature of autolysis with reference to yields.*

Temperature of autolysis	Terminal viable population	Toxin units/ml	Toxin units per 1×10^9 cells of original suspension
4 C	1.3×10^{10}	40	1.3
26	1.7×10^{9}	100	3.3
32	6.1×10^{8}	195	6.5
37	4.0×10^{8}	250	8.3
41	3.2×10^{8}	195	6.5
45	8.5×10^{8}	73	2.4
55	Nil	60	2.0

Organisms grown 4 days at 30 C on agar. Harvested suspension divided and held at various temperatures 48 hours. After centrifugation and filtration the fluids were treated with sodium desoxycholate to a final concentration of 0.5% for 1 hour. Original viable population: 3.1×10^{10} ml.

hours. After centrifuging, filtrates were prepared and these were treated with sodium desoxycholate. The resultant titrations of toxicity, shown in table 3, indicate that maximum was obtained at 37 C. It is probable that the end result is actually an expression of the amount of toxin and precursor released less the amount inactivated at the respective temperatures. It is to be noted that losses in viability do not correspond to the amounts of resultant toxin.

Gaseous environment during growth with reference to toxin yields.—Repeated experiments were carried out to test the possibility that gaseous environment during growth might contribute to the toxin potential. The experiments involved replacement of air in the inoculated Roux bottles by flushing with various gases. The bottles were then sealed and incubated. A summary of the results obtained in the series of experiments is shown in table 4.

Past. pestis grew profusely in the presence of nitrogen. Oxygen provided a slight suppressive influence. Carbon dioxide, on the other hand, provided an atmosphere distinctly inhibitory to vegetative multiplication. However, those cells grown under nitrogen gave very poor yields of toxin, whereas those grown under carbon dioxide had relatively high potentialities.

TABLE 4.—*Toxin yields of agar grown* Past pestis, *with relation to atmosphere.*

Gas	Examples analyzed	Typical viable count	Toxin: Units/10^9 cells	
			Range	Mean
Nitrogen	3	5.6×10^{11}	1.5– 2.3	1.6
Open (Laboratory air)	5	1.1×10^{11}	8.7– 63	28
Oxygen	5	6.5×10^{10}	6.5– 116	64
Carbon dioxide	4	7.3×10^{8}	268 –7150	3271

Roux bottles with agar. After inoculation bottles "flushed-out" with respective gases and closed. 26 C, 3 days. Viable counts per ml harvest fluid (10 ml/Roux bottle). Desoxycholate-type toxin.

Culture mediums with reference to toxin potential.—Repeated attempts have been carried out to learn if growth on certain culture mediums might give yields of toxin related to the composition of the medium. Many mediums do not support the growth of *Past. pestis* sufficiently to permit evaluation but analyses have been carried out with some 50 mediums.

Illustrative of a long series of experiments are 4 which are summarized in table 5. In experiment A, blood agar base was used with various supplements. In B, various standard agars were compared. In C, the type of peptone was varied. In D, static and aerated broth cultures were compared. The original intent, that of supplying known substances as an essential for toxin production, was not achieved. An analysis of these and other experiments, however, points to this conclusion: A medium favorable for vegetative growth frequently gives bacteria with low toxin potentials, and vice versa.

Toxin yields with reference to density of bacterial suspensions.—Examination of all protocols here presented, whether as to culture mediums, temperature of growth or gaseous environment demonstrates that suppression of vegetative multiplication yields bacterial cells of high toxin potentialities. Since the demonstration of this point involved work with suspensions of variable densities of

TABLE 5.—*Culture mediums and the toxin potential of* Past. pestis.

Experiment temperature of growth	Mediums	Viable cells/ml harvest fluid	Toxin units/10^9 viable cells
A 37 C	Blood agar base	1.1×10^{10}	45
	Blood agar base + horse blood	1.2×10^{10}	63
	Blood agar base + yeast extract	4.3×10^{9}	230
	Blood agar base + yeast extract + horse blood	1.5×10^{9}	650
B 30 C	Kligler iron agar	1.5×10^{11}	1.6
	Brain heart infusion agar	1.3×10^{11}	2.8
	Blood agar base	9.1×10^{10}	2.7
	Nutrient agar	8.1×10^{10}	2.8
	Potato infusion agar	3.4×10^{9}	43
	Dextrose proteose agar	2.2×10^{9}	63
C 30 C	Meat extract base + tryptone + agar	4.5×10^{10}	6
	Meat extract base + Bacto peptone + agar	3.1×10^{10}	12
	Meat extract base + neopeptone + agar	1.3×10^{8}	91
	Meat extract base + proteose peptone #3 + agar	6.5×10^{7}	520
D 25 C	Static broth culture (BHI); cells concentrated by centrifugation to 5% of original volume. Desoxycholate (0.5%) 18 hours 4 C. Supernatant fluid	3.2×10^{8}	312
	Aerated broth culture as above	3.6×10^{9}	28

Desoxycholate type toxin from agar grown organisms. Each Roux bottle harvested with 10 ml water.

cells it was important to determine whether cell density during the release of toxic end products might in itself be a contributing factor. This question was particularly important in the instance of the desoxycholate toxin, since it was previously demonstrated that the concentration of the bile salt was an important factor. Hence it was possible that the effect was dependent upon bile salt/bacteria ratio rather than upon the inherent toxigenic potentials of the cells.

Repeated experiments have been carried out with reference to this possibility. All have yielded similiar results. An example is shown in table 6. Organisms were grown at 39 C for 72 hours and harvested in minimal fluid. Fivefold dilutions were then prepared and divided. One set was used for the preparation of lysate toxin, while to the materials of the other set desoxycholate was added (equal volume of 1% sodium desoxycholate).

TABLE 6.—*Concentration of organisms with reference to toxin yields.*

Type of toxin	Bacterial density, initial	Toxin yield, units/ml	Toxin units/10^9 cells
Lysate	3.8×10^{10}	160	4.8
	7.6×10^{9}	33.8	4.4
	1.5×10^{9}	7.9	4.0
Desoxycholate	1.9×10^{10}	503	27
	3.8×10^{9}	108	29
	7.6×10^{8}	22	29

Blood agar base, 72 hours growth at 39 C.

The results show that at each of three population levels corresponding toxin yields were obtained with both lysate and desoxycholate types. The potential then must reside in the cell itself and not in the density of cells yielding the toxin.

DISCUSSION

The suppression of vegetative growth of an avirulent strain of *Past. pestis*, whether by temperature of incubation, nature of culture medium or gaseous atmosphere, provided bacterial cells with higher toxin potentialities. This is of much interest in connection with the study of pneumonic plague by McCrumb et al.[5] In that series of 13 cases blood cultures were uniformly negative. In several instances the amount of pulmonary involvement was not great. There was little evidence of overwhelm-

5. McCrumb, F. R., Mercier, S., Robic, J., Bouillat, M., Smadel, J. E., Woodward, T. E. and Goodner, K. 1953, Chloramphenicol and terramycin in the treatment of pneumonic plague. Am. J. Med. **14**: 284–293.

ing bacterial multiplication, but the patients had high temperatures and were were recorded as "profoundly ill." Since in a subsequent report McCrumb et al[6] have reported that a number of patients developed definite antitoxin during convalesence, it is not improbable that the situation in pneumonic plague may parallel that observed in the laboratory, that is, temperature suppression of vegetative growth with enormous exaggeration of toxigenic potentials.

Englesberg et al[7] have recently reported that virulent strains of *Past. pestis* have, on the average, somewhat higher toxin potentials than avirulent forms. The differences, however, were not great. The present experience has demonstrated that one avirulent strain (E. V. 76) may show very wide differences in toxigenicity dependent upon factors known and unknown. Thus, lysate toxin levels from 0.2 to 134 units/10^9 cells are recorded, and with desoxycholate toxin a range from 1.5 to 7150. It would seem hazardous at this time to attempt to relate toxin potential to virulence.

6. McCrumb, F. R., Mercier, S., Chen, T. H., Foster, L., Larson, A., Meyer, K. F. and Goodner, K. 1955, Studies on the antibody patterns in pneumonic plague patients. J. Infect. Dis. 96: 88–94.
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SUMMARY

Suppression of vegetative multiplication of an avirulent strain of *Pasteurella pestis*, whether by temperature of incubation, adverse culture mediums, or gaseous environment, provided bacterial cells with enormously enhanced toxigenic properties.

EXPERIMENTAL ACTINOMYCOSIS WITH *ACTINOMYCES ISRAELI*

THE DEVELOPMENT OF VISCERAL ACTINOMYCOSIS

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An important step in the elucidation of the mechanism of any infectious disease is the ability to transfer the infection uniformly to a suitable laboratory animal. Such a step has been lacking in the clinical entity, actinomycosis, since irregular results have been obtained in most of the usual laboratory animals.[1,2]

Prior to the work of Meyer and Verges,[3] the use of mice as experimental animals to produce progressive actinomycosis was not studied to any extent. These authors reported that progressive actinomycosis could be almost uniformly produced with *Actinomyces bovis* suspended in hog gastric mucin and injected intraperitoneally into young male white mice (10–15 g). These findings were confirmed by Geister and Meyer,[4] who were able to follow the progression and dissemination of the experimental infection in mice, and were able to assess the prophylactic and therapeutic effectiveness of aureomycin and penicillin against this experimental infection. On the other hand, Strauss and Kligman[5] could not obtain positive results when they injected *A. bovis* in hog gastric mucin intraperitoneally into young male mice, although they were uniformly successful with *Nocardia asteroides* and with the other fungi responsible for systemic mycoses. Hazen, Little and Resnick[6] were able to produce progressive actinomycosis in young male hamsters by intraperitoneal inoculation of *Actinomyces israeli* suspended in saline. The authors suggested that the male hamster be the animal of choice for demonstration of the pathogenicity of *A. israeli*.

The purpose of this investigation was (1) to confirm and extend the findings of Meyer and Verges,[3] in view of the contrary results reported by Strauss and Kligman,[5] and others, and (2) to study the evolution of the experimental infection, histopathologically and bacteriologically.

METHODS AND MATERIALS

Mucin.—The 5% mucin suspensions were prepared according to the method described by Gale and Elberg,[7] using Wilson 1701W granular hog gastric mucin. To 95.0 ml of distilled water in a Waring blendor was added 5.5 g hog gastric mucin. This was allowed to stand 15 to 20 minutes to enable the mucin granules to absorb the water, and blended 5 to 6 minutes until a white, viscous, homogeneous suspension was obtained. The suspension was autoclaved precisely 15 minutes at 121 C (the autoclave brought down very quickly, within 2 minutes) and neutralized with 10 to 11 ml sterile *N*/10 NaOH. Fresh preparations of mucin were always used in the animal inoculations, since standing at room temperature or in the refrigerator tends to aggregate the mucin

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6. Hazen, E. L., Little, G. N. and Resnick, H. 1952, J. Lab. & Clin. Med. **40**: 3–7.
7. Gale, D. and Elberg, S. S. 1952, J. Infect. Dis. **91**: 50–62.

particles and impairs the activity of the mucin suspension.

Mice.—White, Swiss type mice, black C57 mice (both inbred for several years by brother and sister matings), and gray-brown mice (the hybrid produced by crossing the white and black mice) were used. In all the experiments, the mice were randomized according to sex, age and color, in order to test the assertion of Meyer and Verges[5] that old male mice or female mice of any age would give erratic results.

Preparation of the bacterial suspensions for animal inoculation.—The strains of *A. israeli* were grown in 50 ml Erlenmeyer flasks in either BH broth* or in Dubos A medium,† 7 days at 37 C, anaerobically in Brewer jars in an atmosphere of hydrogen and 5% CO_2. The compact "crumbs" were collected by centrifugation at 1500 r.p.m. for 20 minutes, washed 3 times in saline in a graduated 15 ml conical centrifuge tube, and the wet volume estimated. They were transferred quantitatively in a minimal amount of broth to either glass tissue grinders or to Teflon homogenizers, and ground until a homogeneous suspension was obtained (30 to 60 seconds). Sufficient broth was added to make a 2% suspension v/v.

Inoculation of animals.—Mice were injected intraperitoneally by separate inoculations of 0.8 cc of mucin, broth, or saline on one side of the abdomen, and 0.2 cc of the bacterial suspension on the opposite side. This was done in an attempt to ensure uniformity of dosage. It seems doubtful that the mixing of a clumped, branching organism in a viscous medium like mucin would produce a suspension so homogeneous that injected aliquots would contain equal quantities of organisms. By adding the adjuvant and the organism inoculum separately, mixture would be accomplished in vivo by the peristaltic action of the intestinal tract, and by movement of the omentum.

Guinea pigs were inoculated similarly, with 2.0 cc adjuvant and 0.5 cc bacterial suspension, intraperitoneally. For the subcutaneous inoculation, 0.5 cc bacterial suspension was injected in the shaved left flank, and 0.5 cc mucin injected into the same site.

Culture of the organs to recover A. israeli.—Attempts to recover the organism from lesions by shelling out the lesion to streak the surface of a plate, or by streaking the cut surface of the organ, at the lesion site, were not successful. The method adopted follows. The organ or organs containing lesions were removed, pooled, and ground thoroughly with sterile sand and minimal broth in a mortar and pestle, aseptically. Two drops of the resulting tissue suspension removed by capillary pipette were placed on the surface of a BH plate and streaked with a sterile bent glass rod. The plate was incubated anaerobically at 37 C for 7 days. The liver, spleen and omentum were removed, pooled and treated similarly in those animals in which no lesions were observed. It is evident that there are sources of possible error in this procedure, particularly in sampling. In working with a clumping, branching, mycelial organism which may be present in small numbers in the organ or lesion, it may be easy to miss the clumps in the sampling, and thus no growth may appear on the plate. Hence a negative culture result may not reflect the absence of organisms in the lesion.

Histopathology.—Organs containing lesions were fixed in 10% formalin, prepared by paraffin embedding, and stained with hematoxylin-eosin or Gram-Weigert staining procedures.

* Difco brain heart broth.

† Difco TB broth base plus TB medium albumin.

EXPERIMENTAL

Strains of A. israeli *used.*—The seven strains used in this investigation were all from human sources, two from cases of cervicofacial actinomycosis (AN11 and AN14), two from periodontal disease (AN10 and AN9), and three from normal mouths (AN13, DGTS, TRGS).

Five of these strains had been isolated in 1947 and 1948* and were maintained in a dry ice chest in 1.0 ml ampules as described in detail by Rosebury and Francis.[6] When a culture was desired, the appropriate ampule was quickly thawed at 37 C, and the contents transferred by capillary pipette to suitable medium and incubated anaerobically 6 to 7 days at 37 C. To maintain the stock strains, ampules of the subcultures in BH broth were frozen.

The two freshly isolated strains were continuously subcultured as long as they were used, and ampules frozen.

* Graciously contributed by Dr. Theodor Rosebury.

8. Rosebury, T. and Francis, S. 1950, Oral Surg., Oral Med., Oral Path. 3: 1557.

TABLE 1.—*The pathogenicity for mice of various strains of* A. israeli.

Strain and type*	Medium in which grown	Adjuvant injected	Positive reactions†		Gross pathology‡	Positive cultures§
			Lesions	Adhesions		
2% AN9 S	Dubos A	Mucin	6/10‖	3/10	l, m, F	1/10¶
		Broth	4/5	2/5	M	2/5
2% AN10 R	Dubos A	Mucin	8/10	10/10	L, m	6/8
		Broth	5/5	5/5	M, l	2/4
2% AN11 R	BH broth	Mucin	10/10	10/10	L4, m, f	5/6
		Broth	4/5	3/5	M2, l	3/4
2% AN12 I	Dubos A	Mucin	5/7**	4/7	L, m	4/7
		Broth	5/5	4/5	M2	2/5
1.67% AN14 R	BH broth	Mucin	4/10	3/10	L	3/10
		Broth	5/5	5/5	M, l	3/5
2% TRGS S	Dubos A	Mucin	1/10	2/10	L	1/9
		Broth	4/5	1/5	M, l	4/5¶
2% DGTS I	BH broth	Mucin	7/9††	9/9	L3, m	7/9¶
		Broth	4/5	5/5	L, m	4/5¶
No organisms		Mucin	0/5	0/5	none	0/5
		Broth	0/5	0/5	none	0/5

* S, smooth; R, rough; I, intermediate.
† Number of mice showing reactions/number of mice injected, excluding deaths, 24 days after inoculation.
‡ Keyed as follows: capital letters refer to predominant organ showing lesions; L, liver; M, mesenteric organs, great omentum, lesser omentum, mesenteries, falciform ligament; F, fatty tissues; 2, 3, 4, extent of involvement.
§ Prepared from pooled organs on BH plates, incubated anaerobically 7 days; refer to *A. israeli* identifiable by colonial and microscopic morphology.
‖ Includes white areas in fatty tissues, not characteristically lesions.
¶ Includes smooth actinomyces as well as rough actinomyces.
** 3 deaths within 8 days, 2 giving positive cultures for actinomyces.
†† 1 death within 3 days; results shown, 41 days after inoculation.

One of these strains, DGTS, was isolated by the method of Ennever, Robinson and Kitchin.[9] Sediment from tongue scrapings was suspended in dilute Triton A-20 in a micromortar and pestle, ground, and plated serially on BH plates, anaerobically. The other strain, TRGS, was isolated from gingival scrapings on spirochete plates.† No biochemical tests with these strains were done.

Groups of mice were inoculated intraperitoneally with the seven strains of *A. israeli*, together with mucin or broth, respectively. Four of these strains were grown in Dubos A medium, and the other strains were grown in BH broth. Mucin and broth controls were also included. The mice were sacrificed and examined for lesions at various time intervals. All lesions were either cultured or sectioned or both. The results in terms of gross pathology and cultures can be seen in table 1.

All the strains injected with broth as adjuvant produced actinomycosis uniformly, with 88% of such animals showing lesions or adhesions. Variations in pathogenicity could be clearly seen when mucin was used as adjuvant. The smooth strains produced fewer lesions or adhesions than the intermediate or rough strains. The intermediate strains seemed to be as pathogenic as the rough strains. Anomalous lesions were seen in the mice inoculated with AN9, a smooth strain, and mucin. Such lesions were also present, randomly, in animals inoculated with the other strains.

The distribution of lesions in all the mice differed considerably according to the adjuvant used, but was not affected by the strain or the medium in which the strain was grown. The liver was the prime site of lesions in the mucin-in-

9. Ennever, J. Robinson, H. B. C., and Kitchin, P. C. 1951, J. Dent. Res. **31**: 88–97.

† By Dr. Theodor Rosebury, while isolating *Leptotrichia buccalis*.[10]

10. Rosebury, T., MacDonald, J. B., Ellison, S. A. and Engel, S. 1951, Oral Surg., Oral Med., Oral Path. **4**: 68–85.

jected mice, with the possible exception of those animals inoculated with AN9. The mesenteric tissues, including the omentum, were the principal focus of lesions in the broth-injected mice.

Grossly, all lesions were grayish-white, hard, compact nodules, tubercle-like in appearance and could not be easily shelled out from the surrounding tissues. Some of the lesions occurring in the omentum and mesenteric tissues were massive, 50 to 100 mm in diameter. Such lesions were the focus of extensive adhesions involving omentum, spleen, mesenteries, intestine, peritoneal wall and fat bodies.

Pathology.—The microscopic findings in the above animals showed many features in common. The most characteristic process was the formation of single or multiple chronic abscesses, ranging from 10 to 100 mm in diameter. In a typical example, the lesions showed a central collection of polymorphonuclear leucocytes surrounded by a thick wall of granulation tissue. Necrosis was not conspicuous in these lesions. Colonies of the organisms were frequently found in the central area of the abscesses. In sections stained with hematoxylin-eosin, these colonies appeared as slightly basophilic, homogeneous masses. In some cases, definite eosinophilic staining clubs could be seen at the periphery.

With a Gram-Weigert stain, the colonies were seen to be composed of a network of branching Gram-positive filaments. Club formation was present in some sections. The amount of granulation tissue surrounding the central abscess varied with the different sites of the lesions. Abscesses in the liver and, occasionally, in other solid tissues had a wide zone of chronic inflammatory reaction which was rather sharply demarcated from the adjacent compressed parenchyma. This zone was composed principally of fibroblasts, large mononuclear cells and abundant capillaries. Polymorphonuclear leucocytes were scattered through the entire width of this zone. Abscesses in the omentum and mesenteries tended to be less well-defined and had a narrower zone of granulation tissue surrounding the core.

In mice injected with AN9 and mucin, certain atypical lesions appeared quite regularly, although similar lesions were encountered rarely in animals injected with the other strains. These lesions consisted of circumbscribed foci of fat necrosis in the mesenteries and ovarian fat bodies. Histologically, these areas appeared identical with those seen in pancreatic fat necrosis. (The omentum, however, was rarely involved.) The necrotic area was surrounded by a narrow zone of connective tissue. Leukocytic reaction was minimal to absent. Gram-Weigert stains failed to show any organisms.

Atypical lesions were also found in mice injected with the TRGS and DGTS strains. Grossly, the animals showed massive enlargement of the liver. On microscopic examination, the liver was involved by a focal necrotic and granulomatous process. These ill-defined areas consisted of degenerating liver cells, accumulations of mononuclear leucocytes and fibroblasts. Gram-Weigert stains on a number of sections failed to show any organisms in these areas. Typical actinomycotic lesions, however, were also found in some of the mice injected with the DGTS strain.

The development of visceral actinomycosis in mice.—The development of progressive actinomycosis was investigated with particular emphasis on the time of appearance of lesions and adhesions, when one strain of *A. israeli* was injected with various adjuvants. Groups of mice were inoculated intraperitoneally with AN11, BH grown, together

with mucin, broth, or saline, respectively. At daily intervals for the first 10 days, 3 mice in each series for the first 2 days, and 2 mice each for the remaining 8 days were sacrificed and autopsy performed. In addition, at 16, 23, and 29 days after inoculation, 2 mice in each series were also sacrificed and autopsied. Organs showing lesions, or the lesions were removed for anaerobic culture on BH plates, and for sectioning. The heart blood of the 3 mice in each series for the first 2 days, and the 2 mice in each series on the 9th and 16th days, was cultured on BH plates, anaerobically.

The development of the experimental infections, viewed grossly, is presented in table 2. The main points of difference between the mice receiving mucin and AN11 and those injected with broth or saline and organisms can be summarized as follows:

(1) In the mucin-injected mice, lesions were found predominantly dispersed throughout the liver, either visible as small, white, soft areas showing confluent, miliary spreading on the liver surface in the first 2 days, or as many, large discrete, circumscribed lesions of varying size apparent by the 3rd day, and thereafter. The omental and mesenteric tissues were moderately infected, with only sporadic occurrence of massive lesions greater than 30 mm in diameter. In the broth or saline series, only a few lesions were seen in the liver, generally in contact with the diaphragm in the region of the thoracic duct, or at the gallbladder. The major site of infection in these animals was the omentum, both greater and lesser, and the mesenteries, with massive lesions visible by the 5th day in the broth series, and by the 7th day in the saline-injected mice. These lesions were firm, hard and encapsulated with a fibrous envelope, and could not be shelled out of the surrounding tissues.

(2) Lesions were seen in the spleen only in the mucin-injected mice for the first 5 days, although the organ was enlarged in all the mice injected. Spleen involvement occurred in the broth and saline series only in the adhesions formed by the massive lesions.

(3) The sites and frequency of adhesions were quite different in the mucin and the broth or saline-injected mice. In the mucin series, the major lobes of the liver were usually fused together, and could not be separated without tearing the liver substance. A fibrinous

TABLE 2.—*The gross pathology of visceral actinomycosis in mice.*

Day after injection	Liver			Omentum and mesenteries			Spleen			Fibrinous adhesions		
	Mucin	Broth	Saline	Mucin	Broth	Saline	Mucin	Broth	Saline	Mucin	Broth	Saline
1	5*	1	1	0	1	1	2	0	0	a†	a	0
2	5	2	1	1	3	2	3	0	0	b	a	0
3	4	2	1	1	3	2	1	0	0	b	a	b
4	4	2	1	1	3	1	1	0	E	b	b	b
5	4	2	1	1	3 (50)‡	3	1	E	E	b	a	b
6	4	2	1	2	3 (30)	2	E	E	E	b	a	b
7	4	2	2	2	3 (30)	3 (30)	E	E	E	b	c	c
8	3	2	2	1 (75)	2 (40)	2 (30)	E	E	E	c	c	c
9	3	2	3	2 (60)	3 (30)	2 (50)	E	E	E	c	b	b
10	3	2	2	0	3 (60)	2 (30)	E	E	E	b	b	b
16	3	2	2	1	3 (60)	2 (80)	E	E	E	b	b	b
23	3	3	3	1	3 (60)	2 (60)	E	E	E	a	b	b
29	2	1	1	0	3 (50)	2	E	E	E	a	b	a

The strain used was AN11, BH grown.

* Gross pathology keyed as follows: 0, normal; E, enlarged only, no lesions; 1, 1 to 2 lesions; 2, 3 to 6 lesions; 3, 7 to 14 lesions; 4, 15 or more lesions; 5, miliary.

† Adhesions keyed as follows: 0, no adhesions; a, 1 adhesion; b, 2 to 3 adhesions; c, 4 or more adhesions.

‡ 3 (50), 7 to 14 lesions, at least one of which measured 50 mm in diameter.

adhesion of the liver to the diaphragm, which was present in all these mice after the 2nd day, was extensive, involving the anterior flexure of the liver along its entire length. With rare exceptions, all other adhesions observed, involved the liver, adhering either to stomach, omentum, duodenum, or right kidney.

No liver lobe fusion was seen in either the broth or saline series. Liver-diaphragm adhesions occurred only dorsally in the region of the thoracic duct. All other adhesions in these series involved the mesenteric tissues or fatty tissues. The massive lesions mentioned above were the foci of adhesions of the peritoneal wall to omentum to spleen to mesentery to intestine to fat body, in these mice.

Pathologic studies.—The microscopic findings were similar to the results of the gross examination. By the 1st day in the mucin series, a diffuse inflammatory process consisting chiefly of polymorphonuclear leucocytes and fibrin, and involving only the liver capsule was seen. The liver parenchyma seemed essentially normal, although there was an increased number of leucocytes in the sinusoids. In a few areas on the liver surface, localized accumulations of polymorphonuclear leucocytes could be interpreted as early abscesses. There was very little connective tissue reaction about these lesions. In all these areas, colonies of actinomyces could be seen, both by hematoxylin-eosin and by Gram-Weigert stains. No organisms could be seen within the substance of the liver. A similar early lesion was seen to involve the capsule of the spleen. Omental reaction was minimal, with early abscess formation, but no colonies or organisms were visible. By the 2nd day, larger abscesses on the liver surface extended into the liver substance. Necrosis of adjacent liver cells, and early signs of a fibrous capsule were seen, as well as both diffuse and circumscribed inflammatory changes in the connective tissue of the interlobular septa. By the 3rd day and thereafter, chronic abscesses characteristic of actinomycosis were seen in the liver, spleen, and omentum. Sections of the liver of one animal sacrificed on the 23rd day showed multiple lesions with a preponderance of granulation tissue with respect to the number of polymorphonuclear leucocytes. The usual pattern of the typical actinomycotic lesion was lacking. Active fibrosis and repair seemed to be the principal feature.

In the broth series, within the first day, only a small early abscess in the omentum with large actinomyces colonies were seen. The liver surface showed a minimal inflammatory exudate, but no abscesses. Similar lesions were seen on the 2nd day. By the 3rd day and subsequently, typical chronic abscesses were present in the omentum, the liver surface, and in the substance of the liver at the gallbladder region. In older lesions, the zone of connective tissue reaction appeared relatively larger.

The mice injected with organisms and saline showed slower formation and progression of lesions. By the 1st day, an early abscess was seen in the omental fat, with a few typical branching organisms, but no colony. The liver was normal. By the 2nd day, the omental lesions were circumscribed, but did not show the characteristic chronic abscess pattern. By the 4th day, involvement of the liver surface could be seen. Typical lesions were observed in the liver at the gallbladder region on the 5th day. The omental lesions were characteristic chronic abscesses by the 4th day and thereafter. No signs of resolution of the lesions could be seen in a mouse sacrificed on the 23rd day.

TABLE 3.—*The recovery of* A. israeli *by culture from visceral actinomycosis in mice.*

Day after injection	Mucin		Broth		Saline	
	Pooled organs	Heart's blood	Pooled organs	Heart's blood	Pooled organs	Heart's blood
1	2, 2, 1*	2, 0, 0	2, 2, 2	1, 0, 0	2, 2, 2	2, 0, 0
2	2, 2, 2	2, 2, 1	2, 2, 2†	2, 2, 0	2, 2, 2	2, 1, 0
3	2, 0		2, 2		2,† 2†	
4	2, 2		2, 2		2, 2	
5	2, 2		2, 2		2, 2	
6	2, 2		2, 2		2, 2	
7	2, 2		2, 2		2, 2†	
8	2, 2		2, 2†		2, 2	
9	2, 2	1, 0	2, 2	0, 0	2, 2	1, 0
10	2, 2		2,‡ 2‡		2, 2	
16	2, 2	0, 0	2, 2†	0, 0	2, 1†	0, 0
23	2, 2		2, 2		2, 2	
29	2, 2		2, 2		2, 2	

The strain used was AN11, BH grown.
* 0, no organisms; 1, poor growth; 2, good growth.
† Smooth actinomyces in addition to rough actinomyces.
‡ Smooth actinomyces, only, cultured from omental lesions.

Bacteriological studies.—A. israeli was recovered from the pooled organs of all the mice, regardless of the adjuvant used. Smooth actinomyces (non-branching, diphtheroid-like organisms: smooth, entire, buttery colony) as well as rough actinomyces were found in the cultures of some of the mice in each series, seemingly randomly distributed. The 2 mice sacrificed on the 10th day in the broth series yielded only smooth actinomyces from the massive omental lesions.

In addition, clostridial organisms were isolated from many of the mice. These were pleomorphic, Gram-positive, long organisms, showing no spores, but with many curved forms and long filaments. They were not characterized beyond the fact that no stormy fermentation was produced in litmus milk, and they were not pathogenic for mice.

The cultures of the heart's blood showed actinomyces in 3 out of 9 mice by the first day, 7 out of 9 mice by the 2nd day, 2 out of 6 mice by the 9th day, and none out of 6 mice by the 16th day. Although too few mice were used to draw any valid generalizations, actinomyces were recovered from the blood stream to a greater extent in the mucin-injected mice (5 out of 10) than in the broth-injected animals (3 out of 10). A bacteremia due to actinomyces has been described in the literature only once previously.[11]

The development of visceral actinomycosis in the guinea pig.—In order to determine whether similar results could be obtained in the guinea pig under the same conditions as those described above for the mouse, groups of 6 guinea pigs each were inoculated intraperitoneally with AN11 and mucin, broth, or saline, respectively. In addition, 6 guinea pigs were injected with mucin and AN11, subcutaneously. Each guinea pig was weighed prior to the inoculations, and daily for 10 days, to see if there was any relationship between weight loss and the development of lesions. At the various time intervals indicated in table 4, the guinea pigs were sacrificed with chloroform, autopsied, and the lesions cultured and sectioned.

Lesions occurred in the omentum or

11. Northrop, P. M. and Crowley, M. D. 1943, J. Oral Surg. 1: 19–29.

TABLE 4.—*The pathogenicity for guinea pigs of one strain of* A. israeli.

Guinea pig no.	Adjuvant used	Day sacrificed	Gross pathology		Positive culture	Initial weight of guinea pig in g	Maximum weight loss		
			Lesions	Adhesions			Grams	Per cent	Day after injection
136	Mucin, i.p.*	3	1 (150),† omentum	Omentum-diaphragm	Yes	840	98	11.7	2
153		6	1 (30), omentum	Omentum-diaphragm	Yes	692	74	10.7	5
156		9	2, peritoneal wall	Omentum-mesentery, liver-stomach	No	527	105	19.3	5
154		15	None	Uterus-peritoneal wall, Omentum-peritoneal wall	No	713	74	10.4	5
157		22	None	None	No	733	115	15.7	5
155		22	None	None	No	569	89	15.6	5
158	Broth, i.p.	3	3, omentum	None	Yes	510	36	7.1	3
159		6	1 (60), omentum	None	Yes	615	51	7.4	5
142		9	None	None	No	691	36	5.9	2
160		15	None	None	No	590	58	9.8	4
161		22	None	Omentum-peritoneal wall	No	751	53	7.1	2
162		22	None	Omentum-intestine	No	436	56	12.8	2
166	Saline, i.p.	3	1 (60), omentum	Uterus-uterus	Yes	558	65	11.6	3
164		6	1 (10), omentum	None	Yes	662	87	13.1	5
167		9	1 (10), omentum	Omentum-uterus	Yes	654	96	15.1	5
163		15	None	None	No	712	35	4.9	5
165		22	None	None	No	623	60	9.6	3
168		22	1 (90), omentum	None	Yes	627	57	9.1	4
170	Mucin, s.c.	5	1 (150), s.c.	Skin-peritoneal wall		978	77	7.9	5
127		10	1 (90), s.c.	None		955	115	12.0	5
171		10	None	Peritoneal wall-intestine		737	53	7.2	3
169		15	1 (30), s.c.	Skin-peritoneal wall, Liver-intestine		778	28	3.6	2
130		22	None	None		870	54	6.2	4
135		22	None	None		847	62	7.3	5

The strain of *A. israeli* used was AN11, BH grown.
* i.p., intraperitoneally; s.c., subcutaneous.
† 1 (150), one lesion measuring 150 mm in diameter. 1, 2, 3, number of lesions observed.

at the site of inoculation in the guinea pigs injected intraperitoneally, up to the 9th day after inoculation. There seemed to be little difference in reaction, regardless of the adjuvant used. With the one exception, GP #168, in the saline series, no lesions were seen after the 9th day. The liver, spleen, and diaphragm were not infected.

All the guinea pigs lost weight, with the maximum loss occurring by the 5th day. Most of the guinea pigs regained practically all the lost weight by the 9th day. There was no significant* difference (at the 1% level) between the mean percentages of maximum weight lost in all the three series of animals inoculated intraperitoneally. (Mucin series, $13.9 \pm 3.5\%$; broth series, $8.4 \pm 2.9\%$; saline series, $10.6 \pm 3.5\%$.)

The animals injected subcutaneously with AN11 and mucin showed lesions at the site of inoculation, and some adhesions which penetrated the peritoneal wall. No lesions or adhesions were seen after the 15th day. The mean percentage of maximum weight lost, which also occurred by the 5th day, was significantly* different from that of the guinea pigs in which organisms and mucin was injected intraperitoneally. (Mucin series, subcutaneous, $7.4 \pm 2.7\%$; mucin series, intraperitoneal, $13.9 \pm 3.5\%$.)

Microscopically, the lesions were characteristic actinomycotic, chronic abscesses. Signs of resolution of the lesions could be seen in the mucin series at 9 days. The omentum, liver, spleen, and mesenteries were essentially normal

* Using the Student *t* for the difference between means.[12]

12. Fisher, R. A. 1948, Statistical Methods for Research Workers, ed. 10, Edinburgh, Oliver and Boyd, pp. 86.

in the broth-injected animals by the 9th day.

It is apparent that the guinea pig's response to intraperitoneal or subcutaneous inoculation of *A. israeli* was seen only for a short period of time following the inoculation. Repair and resolution of the lesions occurred within 15 days of the inoculation, with rare exceptions.

DISCUSSION

The pathogenicity studies, although limited to 7 human strains, imply that the smooth strains can be differentiated from the intermediate strains on the basis of their pathogenicity for mice when injected together with mucin. Ludwig and Sullivan[13] were able to differentiate the smooth human strains from the bovine strains serologically, although they were identical morphologically.

The uniformity of response in the mice receiving broth as adjuvant (88% infection) as contrasted to the variation in pathogenicity when mucin was injected with *A. israeli* seems a little difficult to explain. A possible answer, however, may be offered when one examines the difference in distribution of the lesions.

It has been shown by many workers (Durham, 1897; Buxton and Torrey, 1906; Dudgeon and Ross, 1906; as cited in Wilson and Miles[14]), that particulate matter injected into the peritoneal cavity is removed by two seemingly opposed mechanisms. One is the aggregation of the particulate matter into masses that are deposited on the surface of the omentum. With movements imparted to the omentum by peristalsis of the intestines, movements of the diaphragm, and the deposition of fibrin on the omental surface, following the inflammatory reaction, the omental folds adhere to each other and trap the aggregated masses. Such aggregates are thereby cleared by means of macrophages and other phagocytes. The other mechanism is the removal of the particulate matter by way of the diaphragmatic lymphatics into the right lymph duct (Durham, 1897[14], Courtice and Steinbeck[15]) or into the thoracic duct. From these lymphatic channels, the particulate matter is carried to the blood stream, and then localized in the liver, spleen and other reticuloendothelial organs where phagocytosis by the fixed macrophages takes place.

The predominant organs showing lesions in the mice injected with broth and actinomyces were the omentum and the mesenteries. The liver showed relatively few lesions, and the spleen no lesions. It is probable that both clearing mechanisms described above occurred. The aggregation of the actinomyces on the omental surface, however, might occur more readily because of the size, clumping and branching of this organism. Moreover, if phagocytosis by the macrophages were inhibited, the organisms might proliferate to form colonies, and the lesions and adhesions described could result. The few lesions in the liver might have occurred by the removal of the actinomyces through diaphragmatic lymphatics and thence to the blood stream, since most of the lesions were found in the anterior flexure of the liver near the diaphragm, or dorsally, in the region of the thoracic duct.

The liver was the primary site of lesions in the animals receiving mucin

13. Ludwig, T. B. and Sullivan, H. R., 1952, Australian J. Exper. Biol. & M. Sc. **30**: 81–93.

14. Wilson, G. S. and Miles, A. A. 1946, Topley and Wilson's Principles of Bacteriology and Immunity, Baltimore, Williams and Wilkins Co., pp. 1040–1041.

15. Courtice, F. C. and Steinbeck, A. W. 1950, Australian J. Exper. Biol. & M. Sc. **28**: 161–169.

and actinomyces. Gale and Elberg[7] have reported that the virulence enhancing activity of mucin may be explained in part by an increase in capillary permeability. Under these circumstances, the organisms would get into the blood stream not only by the normal lymphatic drainage, but also through the capillary walls. Resulting localization in the liver and spleen would occur readily. If phagocytosis by the fixed macrophages were inhibited, proliferation of the actinomyces would result, and the large number of lesions within the liver parenchyma be produced. Localization of actinomyces on the liver surface or omentum or mesenteries may also occur in these mice by the mechanical effects of the viscosity of the mucin, the adsorptive material in the mucin suspension, and by the "fibrin barrier."[7] By multiplication of the organisms in such foci of infection, lesions in these areas would also be seen. It would be anticipated that the larger number of organisms would be carried hematogeneously into the liver parenchyma, because of the increase in capillary permeability by the mucin. Thus, more lesions should be seen in the liver and spleen than in the other visceral organs.

Although the dissemination of actinomyces through the blood stream in man has been postulated by several authors,[16-17] the presence of *A. israeli* in the blood stream has been previously reported only by Northrop and Crowley.[11] They found actinomyces, as well as other indigenous flora in the blood immediately after extraction of a tooth. It is not surprising that *A. israeli* was recovered from the heart's blood of some of our mice on the 1st day after inoculation, since most organisms injected intraperitoneally can be isolated from the heart's blood within 2 to 3 hours after injection (as cited in Wilson and Miles[14].) However, the recovery of actinomyces from the heart's blood 9 days after inoculation was unexpected. It may be that in human pulmonary actinomycosis, the dissemination of the actinomyces might occur by hematogenous spread and localization in the alveoli and bronchioles, rather than by aspiration from the subgingival spaces or from tonsillar crypts. It would be difficult to explain cardiac actinomycosis or bone actinomycosis on a basis other than hematogenous dissemination of the organism.

A possible explanation of the irregular results obtained by earlier workers in their attempts to produce progressive actinomycosis in guinea pigs and rabbits may be that repair and resolution of the lesions may have occurred before the animals were sacrificed and examined for signs of the disease. The mice in all our experiments seemed superficially healthy, despite the massive lesions and visceral involvement described. The guinea pigs lost 10 to 15% of their weight by the 5th day following inoculation, but recovered the weight loss by the 9th day. Few lesions could be found in these animals after the 9th day. Signs of repair or resolution were seen in the mice on the 23rd day, and in the guinea pigs on the 15th day after injection. It is evident that the mouse is a better animal for the production of experimental actinomycosis than the guinea pig.

16. Rosebury, T. in Bacterial and Mycotic Infections of Man, 1952, ed. 2, Philadelphia, J. B. Lippincott Co., Chap. 31.
17. Forbus, W. D. 1943, Reaction to Injury, Baltimore, Williams and Wilkins Co., pp. 759–764.

SUMMARY

Progressive actinomycosis was produced in mice by intraperitoneal inoculation of *Actinomyces israeli* together with mucin or nutrient broth. The

distribution of lesions varied according to the adjuvant used. The liver was the prime site of lesions in the animals receiving mucin, whereas the omentum and mesenteries were the principal foci of infection when broth was used as the adjuvant.

Lesions and adhesions in large numbers in the liver were found in the animals injected with mucin by the third day after inoculation, in the omentum and mesenteries of mice injected with broth and organisms by the fifth day, and in the omentum and mesenteries of mice inoculated with saline and organisms by the seventh day. Actinomyces were readily cultured from all these lesions. The histopathology of these lesions was described.

A bacteremia due to actinomyces was present in almost all the mice examined on the second day after inoculation, and in several mice, on the ninth day after inoculation.

The mouse is preferable to the guinea pig for determining the pathogenicity of *A. israeli*.

A MYCOSIS-LIKE GRANULOMA OF FISH

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Mycoses of systemic distribution are rarely observed in fresh-water fish in this country. In a recent review of atypical cell growths in fishes, Nigrelli[1] cited the only known instance of a mycetoma in a North American fresh-water fish which occurred in the head of fingerling landlocked salmon from an Idaho hatchery.[2] The fungus associated with this granuloma was characterized by a branching septate mycelium. Rucker[3] reported a streptomycete which was pathogenic to blueback salmon. This organism produced internal nodules containing masses of hyphae but no inflammatory response. A pathogenic fungus has been observed frequently in marine fish,[4] however, and in both marine and fresh-water fish in Europe. This organism was tentatively classified as a Phycomycete in or near the order Chytridiales and was assigned to the genus and species *Ichthyophonus hoferi*,[5] later reclassified as *Ichthyosporidium hoferi*.[4] The granuloma described in this report occurs in fresh-water trout and is apparently caused by a budding, yeast-like form with no hyphae which evokes a tremendous inflammatory reaction. Morphologically, the organism does not resemble the previously described Ichthyosporidium. The lesions were first seen accidentally in sections prepared from a diplobacillus infection of brook trout termed "kidney disease." Subsequently, the granuloma was observed in three widely separated infections involving the diplobacillus in each instance.

The histological material was received in a fixed condition; thus, no cultural data were available and the nomenclature and classification of the mycotic organism were not attempted. The present distribution of the disease, however, with its potential threat to domestic fish populations, seemed to warrant a description and discussion of the disease. Efforts are in progress to culture the organism.

MATERIALS

During a 2 year period, the granuloma was diagnosed in tissue sections from fish hatcheries in New Hampshire, New York, and West Virginia. In each case kidney disease was present in epidemic form or had been introduced experimentally.

Tissues were taken from living, moribund fish and fixed in Bouin's solution for 24 hours, then stored in 65% alcohol. Hematoxylin and eosin, Mallory's fungi stain, carbol fuchsin-methylene blue acid fast stain and gram stain were used for all sections.

Case 1.—In the spring of 1952 a serious mortality occurred in the brook trout population of the U. S. Fish and Wildlife Service Station at Berlin, New Hampshire. Individual ponds suffered losses over 50% of their total populations. The disease recurred in 1953 at approximately the same time of year. The mortality reached a peak in early spring and gradually subsided throughout the summer. Affected fish were sluggish, often had distended abdomens, exophthalmos and external blisters on the sides and belly.

Gross examination revealed two characteristic internal lesions. The kidneys were affected to a marked degree by a suppurative-necrotic process

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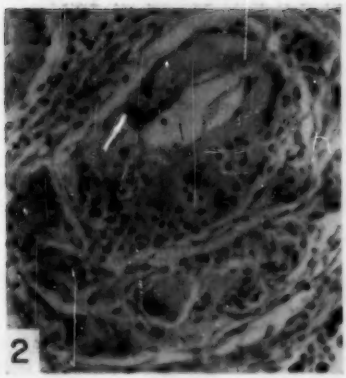
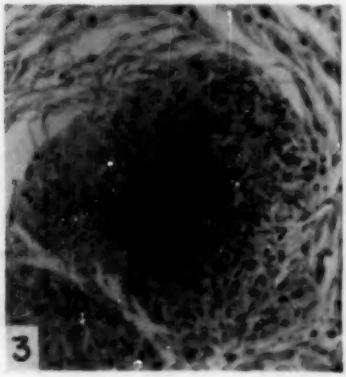
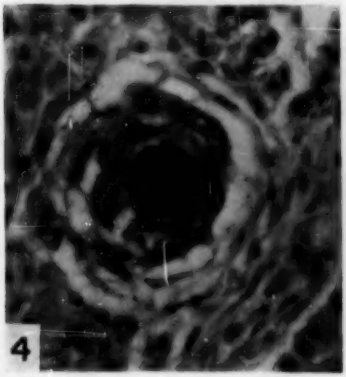






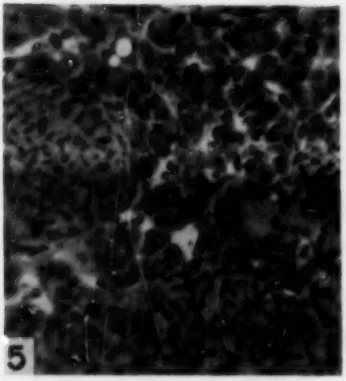
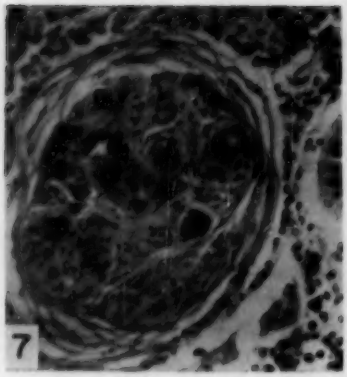
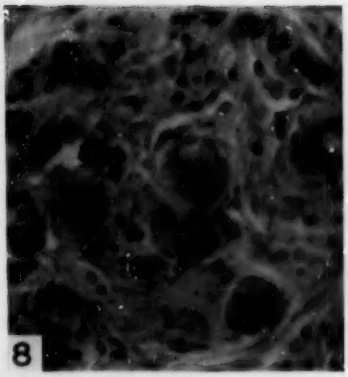
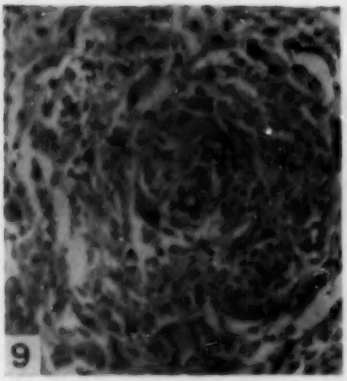
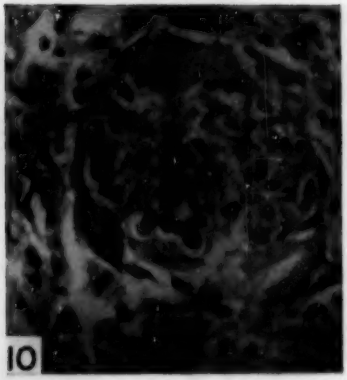


FIG. 1.—Mycotic granuloma. Intestine at lower right. All other tissue is aberrant. ×45.
FIG. 2.—Tubercle in kidney with giant cells. ×200.
FIG. 3.—Typical abscess of visceral lesions. ×200.
FIG. 4.—Small asteroid in center of tubercle. Mallory's fungi stain. ×400.
FIG. 5.—Granuloma in liver from case 2. ×200.
FIG. 6.—Diplobacillus in section at periphery of granuloma. Gram stain. ×2000.









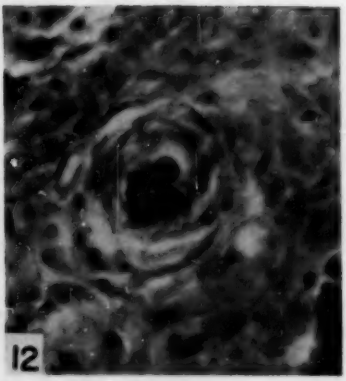


FIG. 7.—Tubercle in kidney with mycotic organism. ×200.
FIG. 8.—Figure 7. ×400.
FIG. 9.—Tubercle in kidney with mycotic organisms and giant cells. ×200.
FIG. 10.—Figure 9. ×400.
FIG. 11.—Mycotic organism in visceral lesion. ×400.
FIG. 12.—Mycotic organism in visceral lesion. ×400.

containing large numbers of gram-positive diplobacilli.[6] The second lesion was represented by a systemic granulomatous reaction most strikingly manifested by masses of aberrant tissue surrounding the gastric caeca and intestines or in extreme cases entirely filling the abdominal cavity. Neither lesion was observed in every moribund fish and both were seen in early stages appearing as slight inflammations.

The rate of mortality subsided after sulfonamide treatments. Gantrisin, sulfamerazine, and sulfamethazine were most effective. However, no treatment was effective in completely eradicating the disease.[6]

Case 2.—In early 1953 material was received from the U. S. Fish and Wildlife Service Experimental Station at Cortland, New York, for diagnosis of an experimentally induced choline deficiency. The granuloma was seen in sections of this material (including the controls) in trace amounts. There was apparently no correlation between the nutritional experiments and the incidence of the granuloma. The diplobacillus was not observed in these sections and no mortality was occurring in the experimental fish at that time. Three months later, the trout population at Cortland suffered a severe mortality. The kidneys were affected by a suppurative necrotic process containing large numbers of the diplobacillus. Grossly, there was no evidence of the granuloma. The large masses of aberrant tissue observed in the New Hampshire fish were not present. Histologically, however, there was an acute granulomatous lesion in the liver which contained the diplobacillus but not the mycotic organism. The mycotic organism was found, however, in trace amounts in the visceral fat surrounding the gastric caeca. Grossly, the disease resembled the epidemic at the New Hampshire Station. The fish exhibited the same symptoms, had approximately the same mortality, and the organism in the kidneys was morphologically similar to the diplobacillus seen previously.

Case 3.—An experimental program was conducted at the U. S. Fish and Wildlife Service Station, Leetown (P. O. Kearneysville), West Virginia, in 1953 to determine the effect of various sulfonamides and antibiotics on the diplobacillus found in the kidneys of diseased fish from Berlin, New Hampshire.[6] To produce a diseased population, individual fish were infected with the diplobacillus by intramuscular or intraperitoneal injection of a suspension of organs of infected fish and placed with the experimental groups. The disease was not transmitted, however, during a 3-week period. The experimental groups were then fed for one day a diet strongly enriched with ground brook trout which had just died or were about to die from kidney disease. Transmission was again unsuccessful. Subsequently, the fish were redistributed, 100 fish per group, and half of each group was infected by parenteral introduction of a freshly prepared suspension from trout with advanced kidney disease. These groups were then treated with various antibiotics. (The results of this series of experiments are published elsewhere.)[6] There was again no transmission of kidney disease to the uninfected half of each experimental group. However, in these uninfected groups subacute stages of the granuloma were present in every group regardless of the medication which they had received. The mycotic organisms were present in large numbers, particularly in the walls of the anterior part of the digestive tract.

The fish which were infected with kidney disease by injection resembled the fish with the natural infections at the New Hampshire and New York stations. The gross pathology and symptoms of the disease were identical and the diplobacillus was recovered from the internal organs of the fish in large numbers.

HISTOPATHOLOGY OF THE GRANULOMA AND KIDNEY LESION

The granuloma, in the presence or absence of the diplobacillus, was typical of the granulomas associated with the various mycoses and tuberculosis of mammals. It was systemic in distribution. In advanced cases, the visceral lesions formed a white, glistening mass which was often mistaken for mature testes. The material was hard and granular, however, due to the tubercle-like nodules which formed a major part of the tissue. In earlier stages, these nodules formed barely perceptible papillae projecting from the outer walls of the stomach or esophagus. Lesions were observed in the muscular wall of the gastrointestinal tract, the adipose tissue surrounding the viscera, the gills, liver, spleen, kidney, eyes, and musculature. No lesions were noted in either the central or peripheral nervous systems. In case 3, which evidently represented a subacute stage of the disease, there was a marked specificity for

6. Snieszko, S. F. and Griffin, P. J. 1955, Prog. Fish. Cult. 17(1): 3–13.

the anterior portions of the gastrointestinal tract. The muscular walls of the esophagus and stomach were practically obilterated by the developing granuloma. Posterior to the juncture of the gastric caeca with the small intestine, there was no evidence of the lesion whatsoever. In this material, scattered lesions were observed in the kidneys and other organs but to a much lesser degree.

The tissue reaction was granulomatous in character. Typical, well-defined tubercles were commonly seen. Giant cells were numerous. In the acute cases, polymorphonulcear leucocytes were the prominent cell type, but leucocyte reaction was negligible in the older, more chronic tissues. In one instance a very marked eosinophilic invasion was observed. When the production of tubercles and granulation tissue was intense in the visceral fat, typical microscopic abscesses were common. As pointed out by Baker[7] this characteristic is typical of blastomycosis.

The organism was a round, budding double-walled, yeast-like form, somewhat resembling the blastomycetes. A heavy capsule was sometimes observed. Endosporulation was not seen nor were hyphae observed. The size ranged from small, single, oval cells of 4 to 5 μ to large, budding cells up to 30 μ. The affinity for hematoxylin was so variable that the darker staining individuals appeared calcified while many forms appeared as "ghosts." The same staining variability was observed with eosin in Mallory's fungi technique.[8]

In advanced cases, yellow, asteroid-like formations were frequently observed. Such asteroids were not stained by eosin but were heavily stained by basic fuchsin. They were rarely over 50 μ in diameter. High magnification failed to resolve any mycelial structures and no organisms were stained within the asteroids by Gram, acid fast, or Giemsa techniques.

When the diplobacillus was present, its location in relation to the granuloma showed considerable variation. In case 2, which was an extremely acute and apparently virulent strain, the bacillus was found throughout the primary stages of tubercle formation, particularly in the liver, but also in the kidney and other visceral organs. In an unusually chronic case from the West Virginia Station, however, the organism was rarely found within the areas of well-defined tubercle formation, but persisted at the periphery of such areas.

The kidney lesion in all cases appeared to be a combination of the granuloma and of a fulminating, massive necrosis attributed to the diplobacillus. In fish, the kidney is the primary site of hematopoiesis, and it was the hematopoietic tissue which was principally affected by the necrosis. The renal epithelium was apparently a secondary victim as the necrosis progressed. It was not unusual, however, to observe normal tubules and glomeruli totally surrounded by completely necrotic tissue.

KIDNEY DISEASE AND THE GRANULOMA

Kidney disease is most prevalent on the Pacific coast of the United States in young salmon. The diplobacillus is morphologically similar to the organism observed in the eastern form of the disease. Earp et al[9] successfully cultured the organism in suspensions of minced chick embryo tissue. Subsequently, the organism was subcultured on Dorset's egg medium. No attempts have been made as yet in the eastern United States to culture the diplobacillus.

Snieszko and Griffin[6] have suggested

7. Baker, R. D. 1942, Am. J. Path. 18: 479–498.
8. Lillie, R. D. 1948, Histopathologic Technic. Philadelphia, The Blakiston Co.
9. Earp, B. J., Ellis, C. H. and Ordal, E. J. 1953, State Wash. Dept. Fish. Spec. Rep. Ser. No. 1, 74 pp., Seattle.

that the eastern and western forms of the disease are identical. Although the organisms of the two forms of kidney disease are morphologically similar, there are some characteristics of the diseases which differ. The western form appears to have a marked virulence in the fall and winter months during a falling water temperature. The eastern form, at least at the New Hampshire Station, is more virulent in the spring during a rising water temperature. Of interest to this discussion, is that the granuloma has not been reported in the western form of the disease by Earp and his associates. In a limited number of histological sections of the western disease, it has not been seen by workers at the Salmon Nutrition Laboratory. In contrast, no eastern form of the disease has been seen without the simultaneous occurrence of the granuloma.

DISCUSSION

There is no experimental evidence to establish a correlation between the granuloma and kidney disease. Indeed it must be acknowledged that the identification of the granuloma as a mycosis is based on the similarity of this disease to those described in mammalian literature. However, the simultaneous occurrence of the two organisms at widely separated points geographically, indicates the possibility of a relationship. There is the possibility of a secondary relationship between the two organisms. It remains to be determined, however, if the diplobacillus is a specific or nonspecific secondary invader after the mycotic organism or vice versa.

It has not been demonstrated that the mycotic organism can be transmitted from one fish to another since in every observation here reported the granuloma may have been introduced by feeding infectious material. It is also possible that all the diseased fish were progeny from the same parent stock at the Berlin, New Hampshire Station, and were shipped as eggs to the New York and West Virginia stations. Likewise, it is not possible to attribute any mortality to the granuloma since large scale losses have always been associated with the diplobacillus. It may be speculated, however, that an insidious disease of this nature may well be correlated with the noted rise of fish mortality in hatcheries of the eastern seaboard over the last ten years.

It is interesting to note a profound change in fish cultural technique which may be a clue to the present observations. Since about 1925, fish producers have added an increasingly greater percentage of dry feed products to their production diets. These ingredients include a variety of the brewer's yeasts, torula yeast (*Torulopsis utilis*, a by-product of the sulphite pulp industry), distiller's solubles, wheat middlings, cottonseed meal, and many types of fish meal. The fish meals are composed of several species of marine fish. To safeguard the nutritional value of these meals, low temperature drying is preferred. It is apparent that a potential source of yeastlike organisms exists. In view of the rather marked distribution of lesions in the anterior portion of the digestive tract, this aspect of the problem deserves further investigation.

SUMMARY

1. A mycosis-like granuloma of fish has been described. The organism somewhat resembles the blastomycetes in tissue sections.

2. A gram-positive diplobacillus is consistently found in necrotic kidney lesions in conjunction with the granuloma.

3. The possible relationship between the mycotic granuloma and diplobacillus is discussed.

THE EFFECT OF *VIBRIO COMMA* MUCINASE UPON THE PERMEABILITY OF MOUSE INTESTINE

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The difficulty in reproduction of cholera in animals has led investigators to study significant features of the pathogenesis of this disease by other methods. Recently attention has centered upon the outpouring of fluid through the intestinal wall and particularly with reference to the matter of "mucinase," an enzyme or enzyme complex described by Burnet and Stone[1,2] and shown by them and by Singer, Wei, and Hoa[3] to produce sloughing of the mucosal cells of the intestinal wall. Burnet[4] and Jensen[5] used ovomucin as a substrate in the evaluation of mucinase—the latter with reference to immunologic aspects. Freter[6] reported that the bowel fluid in experimental cholera in guinea pigs contained mucinase.

The present report deals with the action of mucinase in increasing the permeability of mouse intestine and particularly with the immunologic aspects of this matter.

METHODS

Mucinase.—For the production of mucinase, *Vibrio comma* P93 A was chosen on the basis of previous experience.[5] This culture was maintained on Difco brain heart infusion agar (BHIA). Mucinase was prepared by either of two methods: (a) Brain heart infusion broth (BHI) cultures, grown at 26 C for 18 to 24 hours with forced aeration, were centrifuged to remove most of the cells and the supernate passed through a Mandler filter. The filtrate was stored at −15 C. (b) Cultures were grown on BHIA medium in Petri dishes for 18 hours at 30 C. The agar medium was then transferred to a Büchner funnel overlaid with several thicknesses of washed gauze. A rubber membrane was tied over the opening of the funnel and vacuum applied. The pressure thus exerted squeezed most of the fluid out of the agar. The expressed fluid was filtered through a Seitz pad and stored at −15 C.

The evaluation of mucinase content followed the methods described by Burnet[2] and Jensen.[5] These methods are considered too crude to warrant reliance as to quantitative interpretation.

Immunization of mice.—Vaccines were prepared from 24-hour, 30 C BHIA cultures by suspending washed cells in 1:1000 merthiolate for 24 hours and then diluting the suspension so that it contained approximately 900,000 organisms per ml and a final concentration of merthiolate of 1:100,000. Mucinase having a titer of 2400 ovomucin units was preserved in a merthiolate saline mixture with a final merthiolate concentration as above. This was used as an immunizing agent alone or in combination with an equal amount of the vibrio vaccine. Immunization of mice involved 3 intraperitoneal injections of 0.2 ml at 3-day intervals. Each immunization procedure was carried out on 6 mice which were then used for testing 7 to 15 days after the last injection.

Alterations in intestinal permeability.—Mice which had been without food for 24 hours were

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2. Burnet, F. M. 1948, The mucinase of *Vibrio. cholerae.* Australian J. Exper. Biol. & M. Sc. **26**: 71–80.
3. Singer, E., Wei, S. H. and Hoa, S. H. 1948, Immunological studies of cholera filtrates. J. Immunol. **59**: 341–348.
4. Burnet, F. M., Ovomucin as a substrate for the mucinolytic enzymes of *Vibrio cholerae* filtrates. Australian J. Exper. Biol. & M. Sc. **27**: 245–252.
5. Jensen, K. E. 1953, Immunologic characterization of a mucinolytic enzyme of *Vibrio cholerae.* J. Infect. Dis. **93**: 107–110.
6. Freter, R. 1954, Fatal enteric cholera infection in the guinea pig. Bact. Proc., p. 56.

sacrificed and the small intestine was removed. A tapered glass cannula 8 cm long was inserted into the duodenal end of the intestine. This was secured in place by means of two ligatures. A blunt needle was introduced into the lumen of the distal end of the intestine and a tight ligature applied at this point to prevent the loss of material during the instillation. By means of a tuberculin syringe, 0.6 ml of the preparation to be tested was introduced through the needle. The ligature was tightened after the needle was withdrawn and a second one applied below the first. The test material and the intestinal contents were mixed and distributed in the lumen by raising and lowering the section of the intestine several times. The intestines were then placed in a beaker containing Tyrode's solution to a depth of 7 cm and maintained at 37 C in a water bath. The free end of the cannula was attached to a manometer by means of a short length of heavy walled rubber tubing. The S-shaped manometer, 26 cm in height, was constructed of capillary glass tubing having a 1.5 mm bore. A 3-way stopcock was attached to the lower aperture to permit equilibration. The manometer fluid was water containing small amounts of sodium glycocholate and methylene blue. At the time of attachment the stopcock was in the open position to permit equilibration. The stopcock was then closed and the subsequent changes in the height of the manometer fluid were observed. Successive observations were made as required for about an hour. As many as 8 preparations could be carried out in a single experiment.

A second experimental method involved ob servations on the intestine in situ. Mice were anesthetized with ether and the intestines exposed by abdominal incision. Care was taken to avoid excessive bleeding. A "V" shaped cut was made in the intestinal wall at the duodenum with the vertex pointing toward the lower intestine. A wetted cannula as above was placed in the opening and securely tied in place with the threads previously placed in position. The cannula was connected to the manometer as before. A ligature was now applied at the cecal junction and the test material (0.6 ml) introduced into the lumen of the intestine just above this ligature by means of a syringe. Precautions were taken to avoid leakage as with the excised intestine. The system was allowed to equilibrate for 2 minutes and manometric determinations made as before. A piece of gauze moistened with Tyrode's solution was placed over the intestines to avoid drying of the membranes.

Demonstration of increased permeability.—The results of a typical experiment with substances introduced into the lumen of the isolated mouse ileum are summarized in figure 1. It is to be noted that the mucinase-containing filtrate gave an impressive change in the permeability of normal mouse intestine. The pressure change, interpreted as fluid intake, became appreciable within 15 minutes after first observation and the gain was almost linear up to 30 minutes. Attention should be called, however, to the fact that the time lag was actually somewhat longer than shown, for there were short technical time requirements for installation and equilibration. The figure also shows that heat-inactivated filtrate elicited no change in the pressure of fluids in the lumen of the intestines. This was not always the case with heat-inactivated material, but the change seldom exceeded 5 mm in manometric height in the period of observation, whereas with active mucinase the change was always much greater.

The preparations of intestine were weighed before and after each experiment. In each instance in which a rise of pressure had been observed it was found that there was an associated weight gain of 0.2 to 0.4 g, a finding interpreted also as indicating fluid intake. Intestines into which active enzyme was introduced continued to show active peristaltic movements but tended to develop a transparency as compared to preparations containing inactive materials.

These results, typical of many experiments, are interepreted as showing that mucinase had altered the permeability of the intestine in such a manner as to permit the internal hydrostatic pressure to reflect eventually the external pressure, whereas in intestines not treated with mucinase the effect of the external hydrostatic pressure was slight. This alteration in permeability may represent a situation more complicated than

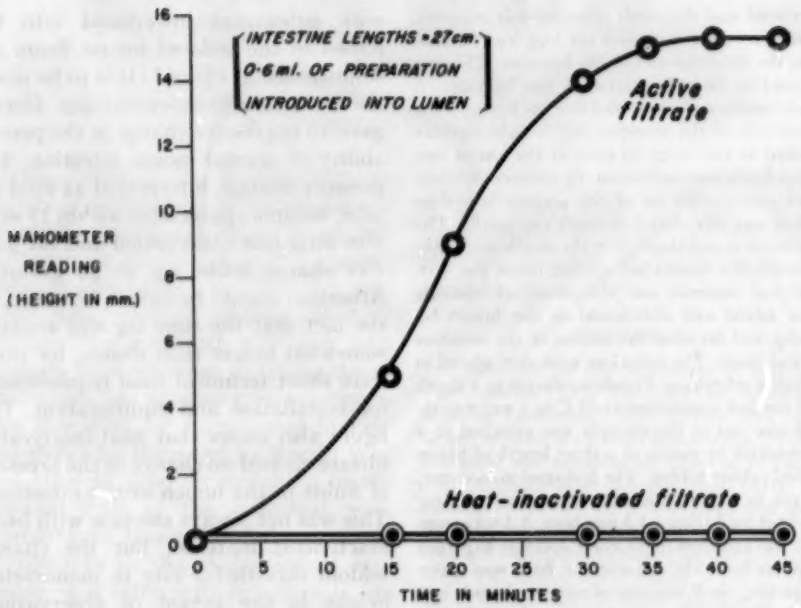

FIG. 1.—The effect of *Vibrio comma* mucinase on the permeability of isolated mouse intestines. Changes in permeability measured manometrically.

that of desquamation of mucosal cells or of depolymerization of intestinal mucous.

The effect of passive immunization.—Mice were passively immunized with 0.5 ml of rabbit anti-mucinase serum intraperitoneally. The serum had previously been tested for neutralizing power against mucinase by the clot inhibition technic and found to be of high titer. The mice were sacrificed 24 hours after the administration of the serum and their intestines subjected to the experimental procedure. Representative results from such experiments are shown in figure 2. It may be noted that the intestine from a mouse passively immunized to mucinase showed no significant increase in permeability after the introduction of mucinase. Repeated experiments on intestines of passively immunized mice under similar experimental conditions gave results comparable to those obtained with non-immunized mouse intestines treated with inactive filtrate. The intestines of passively immunized mice, tested with heat-inactivated mucinase, gave no significant pressure change.

The effect of active immunization.—The fact that passive immunization against mucinase furnished protection to the intestine against the action of mucinase led to the question of relative value of various antigenic preparations.

Three groups of animals were immunized as follows: group I received washed chemically killed cells of *V. comma*, group II, mucinase alone, group III, a combination of mucinase and *V. comma* cells. A fourth group served as controls. Results obtained with this type of experiment are illustrated in figure 3.

It is to be noted that the intestines of mice actively immunized with *V.*

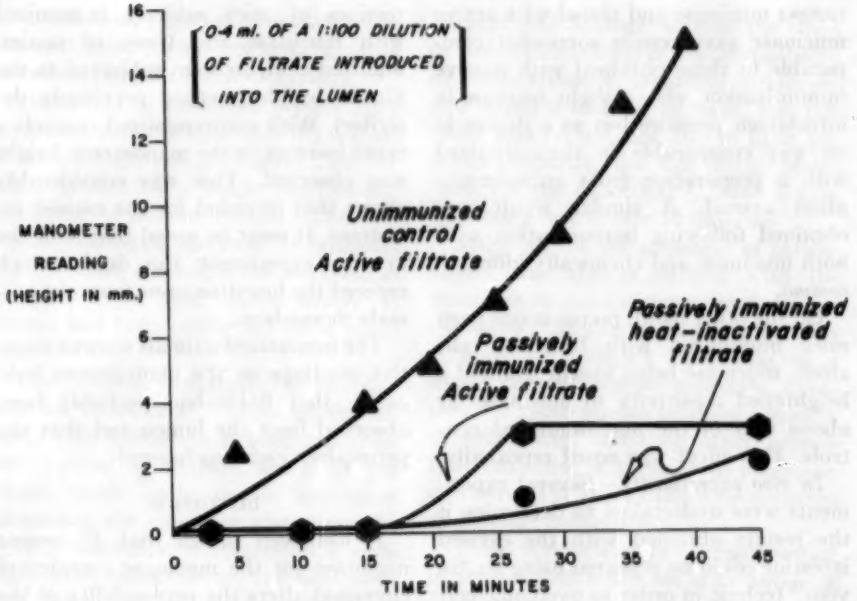

FIG. 2.—The effect of *V. comma* mucinase on the permeability of isolated intestines of mice which had been passively immunized with antimucinase serum.

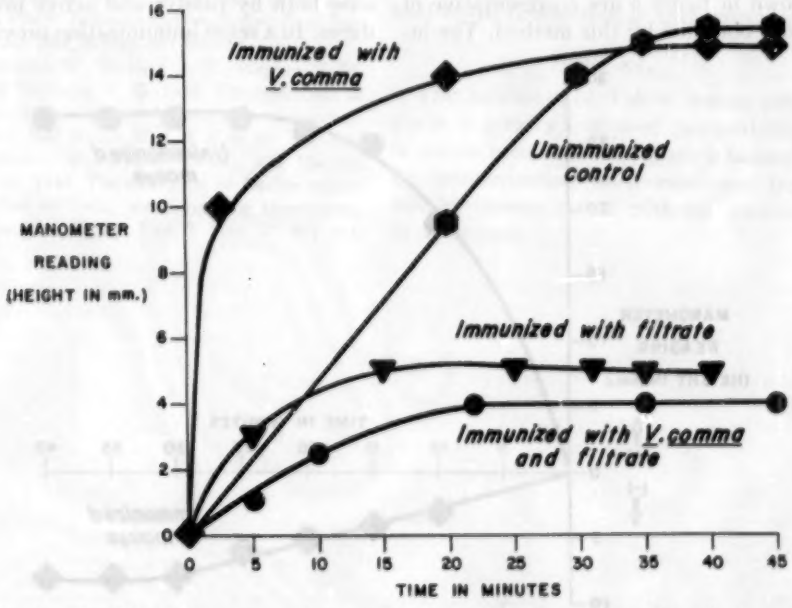

FIG. 3.—The effect of *V. comma* mucinase on the permeability of isolated intestines of mice actively immunized with *V. comma* and with mucinase.

comma mucinase and tested with active mucinase gave results somewhat comparable to those obtained with passive immunization, viz., a slight increase in intralumen pressure but to a degree in no way comparable to that obtained with a preparation from an unimmunized animal. A similar result was obtained following immunization with both mucinase and chemically killed *V. comma*.

On the other hand, preparations from mice immunized with bacterial cells alone, mucinase being absent, showed a heightened sensitivity to mucinase far above that of the nonimmunized controls. This effect was noted repeatedly.

In vivo experiments.—Several experiments were undertaken to determine if the results obtained with the excised intestine could be repeated using an "in vivo" technic in order to overcome certain objections to the artificial nature of the "in vitro" technic. The results shown in figure 4 are representative of those obtained by this method. The intestines of mice actively immunized with mucinase and those of nonimmunized controls were subjected to the filtrate by the method previously described. With nonimmunized controls a rapid increase in the manometric height was observed. This was considerably above that recorded for the excised intestines. It must be noted that with the in situ experiment the fluids which entered the intestine came from the animals themselves.

The immunized animals showed negative readings on the manometers indicating that fluids had probably been absorbed from the lumen and that the permeability was unchanged.

DISCUSSION

It has been shown that *V. comma* mucinase (or the mucinase complex of enzymes) alters the permeability of the mouse intestine but that this effect is blocked by immunization against mucinase both by passive and active procedures. In a sense immunization provides

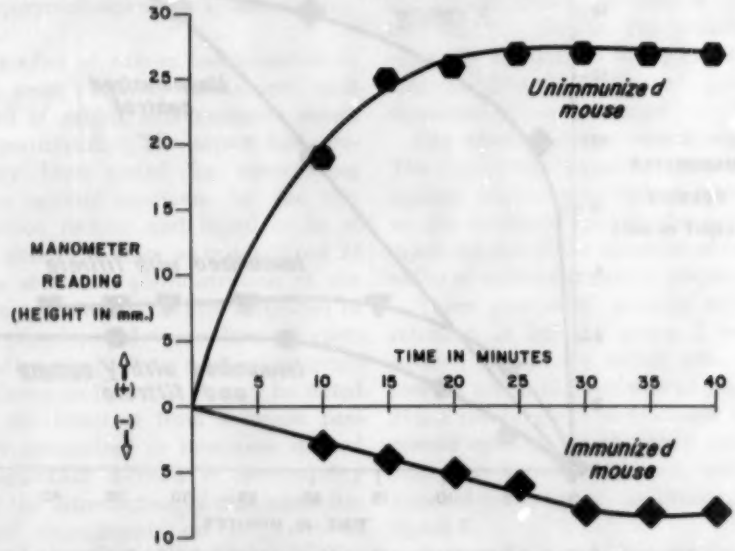

FIG. 4.—The effect of *V. comma* mucinase on the permeability of mouse intestine in situ.

a "protection" against mucinase. The demonstration was convincing with intestines in situ as well as excised.

This effect of enhanced permeability is parallel with the most striking feature of the pathogenesis of cholera. That the effect is due to a single enzyme-like substance is not as evident. The "receptor destroying enzyme" is probably not involved, since it is not obtained in quantity from agar upon which *V. comma* has been cultivated as is mucinase.

The "cholera endotoxin" of Burrows and his coworkers[7–9] must be considered. This endotoxin is prepared from cell lysates, a method which in our hands yields little or no mucinase. Moreover, the mucinase system, unlike that of the toxin, is extracellular, nondialyzable, insoluble on the acid side, and insoluble in alcohol. Burrows, Wagner and Mather[9] studied the effect of the endotoxin upon the permeability of various tissues, including intestines, and found a marked increase, but this effect was rather slow and of little importance during the first hour. Mucinase, on the other hand, gives a pronounced effect within 30 minutes.

Although the enhancement of permeability was always parallel with the mucinase content as determined against ovomucin, the change in all probability is more complicated than that of a single enzyme, single substrate situation, for these vibrios produce a variety of substances, including other proteolytic enzymes, in addition to mucinase, and several of these cannot be excluded as possible participants.

These results support the suggestion of Jensen[5] that mucinase in an antigenic form might well be incorporated in cholera vaccines, since immunization with the usual cholera vaccine does not lead to the production of anti-mucinase.

SUMMARY

The mucinase of *Vibrio comma* produces a greatly increased permeability of mouse intestine. This effect is blocked by immunization with mucinase but not by immunization with suspensions of *V. comma*.

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THE UTILIZATION OF KREBS CYCLE INTERMEDIATES BY THE CULTURE FORMS OF *TRYPANOSOMA CRUZI* AND *LEISHMANIA TROPICA*

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Recent studies on homogenates of the culture form of *Trypanosoma cruzi* have shown the presence of several dehydrogenases (succinic, malic, fumaric) belonging to the tricarboxylic acid cycle (Baernstein, 1953; Seaman, 1953). Since this parasite alternates between two hosts, the mere finding of such enzymes, though strongly suggestive, does not prove necessarily their activity in vivo. They could, theoretically, be a reserve for a changed metabolic pattern upon changing hosts. It seemed therefore of interest to approach the question from another angle, studying whether intact organisms are capable of utilizing typical intermediates of the Krebs cycle. Parallel determinations were done with the culture form of *Leishmania tropica*, because the latter appears in culture in the leptomonad form while in *T. cruzi* cultures crithidial forms predominate.

MATERIALS AND METHODS

The parasites (*T. cruzi* Brazil strain, *L. tropica* Teheran strain) were grown in 250 ml Erlenmeyer flasks on the diphasic medium of Johnson (1947) at 24 C. *T. cruzi* was harvested 7 to 12 days, *L. tropica* 4 to 7 days after inoculation. The overlay of several flasks was pooled and the organisms concentrated by 10 minutes centrifugation at 2500 G. They were then washed twice with either Ringer's solution or isotonic KCl solution and were then suspended in an appropriate amount of one of these solutions.

The rate of oxygen consumption was determined at 30 C by the standard Warburg technique in vessels of about 15 ml capacity containing 1.0 ml parasite suspension, 60 micromoles of phosphate buffer giving a final pH of either 7.0 to 7.4 (average 7.2), or 5.2 to 5.8 (average 5.6), 5 to 20 micromoles of substrate (details under *Results*), Ringer's solution or isotonic KCl solution to give a total volume of 2.0 ml. Control vessels without substrate were used to determine the endogenous rate. Each determination was done in duplicate and each experiment was repeated at least 3 times. The experiments lasted 2 hours and readings were taken at 15-minute intervals.

In some experiments substrate disappearance was followed by determining initial and final levels by the following methods: pyruvic and α-ketoglutaric acid according to Friedeman and Haugen (1943), fumaric acid by determining the optical density at 240 mμ according to Racker (1950).

We are indebted to Dr. E. Bueding for a specially purified sample of sodium pyruvate. D,L-iso-citric acid was prepared from D,L-iso-citric acid lactone (California Foundation for Biochemical Research) according to the procedure of Krebs and Eggleston (1944). The other substrates used were samples of highest purity obtainable from commercial sources.

The nitrogen content of the parasite suspension was always determined in an aliquot by a micro Kjeldahl procedure.† It was found that in both species 1.0 mg N corresponded rather closely to 1 billion organisms. The nitrogen concentration per Warburg vessel varied between 0.2 and 1.3 mg N in the respiratory experiments and between 0.8 and 1.3 in the experiments dealing with substrate disappearance.

RESULTS

Both *T. cruzi* and *L. tropica* survived equally well in Ringer's solution and in isotonic KCl. The latter medium was included because it is frequently employed in experiments with homogenates

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† We are indebted to C. Elwood Claggett for performing the nitrogen determinations.

TABLE 1.—*Influence of Krebs cycle intermediates on the oxygen consumption of* T. cruzi.

Substrate, final molarity	pH	In Ringer's solution		In isotonic KCl solution	
		Oxygen consumption in percent of endogenous rate	Substrate rate minus endogenous rate mm³ O_2/mg N/hour	Oxygen consumption in percent of endogenous rate	Substrate rate minus endogenous rate mm³ O_2/mg N/hour
Pyruvate 0.0025 *M*	7.2	122	21	145	65
Pyruvate 0.0025 *M*	5.6	121	25	154	70
Citrate 0.01 *M*	7.2	100	0	110	16
Citrate 0.01 *M*	5.6	111	18	123	31
Cis-aconitate 0.01 *M*	7.2	101	2	115	9
Cis-aconitate 0.01 *M*	5.6	106	9	143	19
Iso-citrate 0.01 *M*	7.2	101	1	98	−2
Iso-citrate 0.01 *M*	5.6	125	43	159	25
α-ketoglutarate 0.01 *M*	7.2	100	1	114	19
α-ketoglutarate 0.01 *M*	5.6	127	29	126	34
Succinate 0.01 *M*	7.2	111	13	110	5
Succinate 0.01 *M*	5.6	150	57	189	103
Fumarate 0.005 *M*	7.2	99	−2	113	14
Fumarate 0.005 *M*	5.6	106	8	108	9
Malate 0.01 *M*	7.2	99	1	108	12
Malate 0.01 *M*	5.6	136	43	146	58

Endogenous rates in mm³ O_2/mg N/hour: in Ringer's solution, pH 7.2: 133 ± 8.6, pH 5.6: 128 ± 8.1; in isotonic KCl, pH 7.2: 118 ± 10.8, pH 5.6: 110 ± 10.8.

and cellular fractions. It seemed of interest to ascertain whether the ionic environment has an appreciable influence on the metabolism of entire organisms. The endogenous oxygen consumption of *T. cruzi* was not significantly altered either by a change in pH or ionic environment (table 1). In *L. tropica*, on the other hand, the endogenous rate was identical in both salines only at low pH. At pH 7.2 it was markedly lowered in Ringer's solution, but distinctly enhanced in KCl (table 2). This resulted in the endogenous rate being twice as high at pH 7.2 in isotonic KCl than in Ringer's solution. Although this differential effect is difficult to understand, it is known that the potassium ion frequently stimulates respiration (e.g., *Eustrongylides*, von Brand, 1943).

The change in the rate of oxygen consumption of *T. crusi* (table 1), under the influence of various substrates, was essentially the same in Ringer's solution and in KCl. A stimulation above the endogenous level occurred regularly at the lower pH level. The stimulation was not very pronounced with some of the

TABLE 2.—*Influence of Krebs cycle intermediates on the oxygen consumption of* L. tropica.

Substrate, final molarity	pH	In Ringer's solution		In isotonic KCl solution	
		Oxygen consumption in percent of endogenous rate	Substrate rate minus endogenous rate mm³ O_2/mg N/hour	Oxygen consumption in percent of endogenous rate	Substrate rate minus endogenous rate mm³ O_2/mg N/hour
Pyruvate 0.0025 *M*	7.2	99	−1	103	6
Pyruvate 0.0025 *M*	5.6	100	−3	116	21
Citrate 0.01 *M*	7.2	180	59	101	−1
Citrate 0.01 *M*	5.6	159	32	126	30
Cis-aconitate 0.01 *M*	7.2	184	59	100	0
Cis-aconitate 0.01 *M*	5.6	162	43	130	32
Iso-citrate 0.01 *M*	7.2	246	71	101	0
Iso-citrate 0.01 *M*	5.6	177	63	162	74
α-ketoglutarate 0.01 *M*	7.2	197	78	96	−8
α-ketoglutarate 0.01 *M*	5.6	136	52	131	34
Succinate 0.01 *M*	7.2	204	58	101	1
Succinate 0.01 *M*	5.6	180	91	140	63
Fumarate 0.005 *M*	7.2	143	39	99	−3
Fumarate 0.005 *M*	5.6	128	40	134	41
Malate 0.01 *M*	7.2	164	39	99	−2
Malate 0.01 *M*	5.6	132	30	140	41

Endogenous rates in mm³ O_2/mg N/hour: in Ringer's solution, pH 7.2: 69 ± 8.1, pH 5.6: 113 ± 10.0; in isotonic KCl: pH 7.2: 159 ± 12.9, pH 5.6: 115 ± 7.2.

substrates, but since it was found in every experiment, it is considered significant. At pH 7.2 the respiration, in the presence of most substrates, was practically identical with the endogenous rate. However, in a few instances, such as with added pyruvate, it was also increased. *L. tropica* (table 2) showed a similar behavior only in KCl. In Ringer's solution its respiration was stimulated by the various substrates as much, if not more, at pH 7.2 than at pH 5.6. With *L. tropica*, however, the addition of pyruvate did not lead to an increase in respiratory rate in Ringer's solution.

The data on substrate utilization (table 3) show that pyruvate was utilized at both pH levels, irrespective of whether it stimulated the respiration or not. *T. cruzi* utilized larger amounts than *L. tropica*. In our first experiments, which are not included in the tables, we used a commercial sample of sodium pyruvate. It was readily utilized by *T. cruzi* but proved highly toxic to *L. tropica*. A final concentration of 0.01 *M* killed the organisms rapidly and even as low a concentration as 0.0005 *M* inhibited the respiration by about 30%. The sample evidently contained an impurity highly toxic to *L. tropica* but innocuous to *T. cruzi*. A parallelism to this finding is that erythrocyte-free *Plasmodium lophurae* also is exceedingly sensitive to impurities of pyruvic acid (Bovarnick, Lindsay, and Hellerman, 1946). Whether this impurity is identical with that recently described by Montgomery and Webb (1954) has not been determined.

Another curious difference between the two organisms was found when studying the disappearance of fumarate. Deproteinized samples of *L. tropica* to which no fumarate had been added, showed practically no absorption at 240 mμ, the wave length at which fumarate strongly absorbs. Similarly treated samples of *T. cruzi* regularly showed a considerable absorption. Furthermore, this absorption decreased markedly after the 2-hour incubation period, indicating that some endogenous material absorbing at this wave length was consumed. The nature of this endogenous material is unknown at present since many natural compounds, e.g., nucleotides, also absorb at this wave length. The figures shown in table 3 for the fumarate consumption of *T. cruzi* were calculated on the unproven assumption that the endogenous consumption was unaffected by the addition of fumarate. It will be noted that apparently more fumarate disappeared

TABLE 3.—*Relations between substrate utilization and oxygen consumption.*

Species	Substrate	pH	Substrate utilized μg/mg N/hour	Total oxygen consumed mm^3/mg N/hour	Substrate rate minus endogenous rate mm^3 O_2/mg N/hour	Oxygen required for total oxidation of utilized substrate mm^3/mg N/hour
T. cruzi	Pyruvate	7.2	154	125	23	98
T. cruzi	Pyruvate	5.6	215	119	10	136
L. tropica	Pyruvate	7.2	7	102	3	4
L. tropica	Pyruvate	5.6	70	78	−3	45
L. tropica	Pyruvate*	7.2	19	155	6	12
L. tropica	Pyruvate*	5.6	80	141	21	51
T. cruzi	α-ketoglutarate	7.2	6	115	−14	4
T. cruzi	α-ketoglutarate	5.6	70	133	22	43
L. tropica	α-ketoglutarate	7.2	0	106	13	0
L. tropica	α-ketoglutarate	5.6	27	97	9	17
T. cruzi	Fumarate	7.2	48	163	−3	28
T. cruzi	Fumarate	5.6	15	166	3	9
L. tropica	Fumarate	5.6	50	128	27	29

* In isotonic KCl solution; all other data from experiments in Ringer's solution.

TABLE 4.—*Release of malonate inhibition by various substrates.*

	T. cruzi		*L. tropica*	
	Oxygen consumption		Oxygen consumption	
	Percent of endogenous rate	Total minus endogenous mm^3/mg N/hour	Percent of endogenous rate	Total minus endogenous mm^3/mg N/hour
Endogenous + malonate	65		36	
Endogenous + succinate	142	74	140	56
Endogenous + succinate + malonate	133	119	135	137
Endogenous + malonate	65		29	
Endogenous + malate	125	44	117	28
Endogenous + malate + malonate	105	70	36	10
Endogenous + malonate	65		29	
Endogenous + α-ketoglutarate	138	66	122	35
Endogenous + α-ketoglutarate + malonate	76	20	39	15
Endogenous + malonate	65		65	
Endogenous + pyruvate	175	132	94	−7
Endogenous + pyruvate + malonate	128	109	62	−3
Endogenous + malonate	41		36	
Endogenous + fumarate	107	10	125	35
Endogenous + fumarate + malonate	49	11	58	30

All experiments in Ringer's solution. Final malonate concentration: 0.005 *M*. Final substrate concentration: 0.01 *M*, but fumarate 0.005 *M*.

at pH 7.2 than at pH 5.6. This proportion may well be wrong, if fumarate should replace more of the endogenous consumption at the low than at the high pH level.

Malonate strongly inhibited the endogenous respiration of both *T. cruzi* and *L. tropica* (table 4), and this inhibition was completely released by added succinate. It was also largely released by malate in *T. cruzi* but not in *L. tropica*. The other substrates tested did not release the malonate inhibition of the endogenous respiration, but they were still utilized at the same or at a somewhat reduced rate than in the absence of the inhibitor.

DISCUSSION

The experiments summarized above have shown that added intermediates of the tricarboxylic acid cycle increased the respiration of the culture form of *T. cruzi* and *L. tropica* over the endogenous rate. In most series this stimulation took place only at low pH, undoubtedly due to the better permeability of the acids in question in their anionic form. However, the oxygen consumption of *L. tropica* was also stimulated at pH 7.2 in Ringer's solution. It is problematical whether this indicates ready permeability of the acids at this pH. An alternative explanation could be that only small amounts penetrated the bodies of the flagellates and "sparked" the endogenous metabolism. This latter view finds some substantiation in the observation that the actual utilization of pyruvate and α-ketoglutarate was higher at low pH, although the respiration was raised to approximately the same degree at both pH levels. In fact, no α-ketoglutarate utilization could be demonstrated at pH 7.2, but this may have been due, in part, to the relative insensitivity of the method.

The experiments discussed above, coupled with the finding that malonate inhibits the endogenous respiration to a marked degree, suggest strongly that a cycle corresponding to the Krebs cycle is operative in both organisms. It must be assumed, however, that the cycle is not equally active in all its steps. This is indicated by two observations. First, both *T. cruzi* and the leishmanias excrete rather large amounts of succinic acid when carbohydrate serves as substrate (Chang, 1948; Baernstein, 1953; Crowther, Fulton, and Joyner, 1954). Second, it has been shown above that

upon simultaneous determinations of the respiratory rate and substrate disappearance, more substrate disappeared than corresponded to the excess oxygen consumption, if complete oxidation of the substrate is assumed. This may indicate incomplete oxidation of the substrates offered. It could be interpreted as substitution of substrate oxidation for endogenous respiration, but this interpretation is less likely because our malonate experiments seem to indicate that substrate oxidation is essentially superimposed on endogenous oxidation.

The fact that a relatively small excess of succinate completely releases the malonate inhibition is worthy of note (table 4). The phenomenon is more pronounced than that found in *Chilomonas* (Holz, 1954) but comparable to the finding of Seaman (1949) for *Colpidium*. Actually, the amount of oxygen attributable to the oxidation of added succinate was greater in the presence than in the absence of malonate, if the assumption is made that the addition of the substrate does not change the inhibition of the endogenous respiration. However, the strong inhibition of the endogenous respiration by malonate and the relative specificity of malonate inhibition may indicate that part of the endogenous respiration is due to the oxidation of endogenously formed succinate. If this assumption is correct, our observations do not indicate that the succinate oxidation proper is insensitive to malonate. They merely show that a relatively small surplus of succinate is sufficient to dislodge the inhibitor from the enzyme.

Chang (1948) reported that *T. cruzi* and the leishmanias produce succinic and other acids during growth, but that these acids disappeared again in older cultures. He concluded that this was due, at least in the case of *T. cruzi*, to the appearance of morphologically distinct forms for which he assumes different metabolic characteristics. However, the present study has shown that parasites from young cultures consume succinic acid when the medium does not contain carbohydrate and has an appropriately low pH. Since in cultures of *T. cruzi* the glucose rather rapidly is exhausted, and the medium becomes acid (von Brand, Tobie, Kissling, and Adams, 1949), it is evident that then conditions are suitable for a reabsorption of succinic acid even by parasites that in the earlier stages of the culture had produced it. It is hence not necessary to postulate a fundamental change in metabolic pattern.

SUMMARY

1. The culture forms of *Trypanosoma cruzi* and *Leishmania tropica* showed an increased respiration when the medium contained intermediates of the Krebs cycle.

2. Direct determinations revealed the consumption of pyruvate, α-ketoglutarate, and fumarate, more substrate disappearing than corresponded to the excess oxygen consumption.

3. Malonate inhibited strongly the endogenous respiration of both species, and this inhibition was released by succinate. It is concluded that the parasites probably have a sequence resembling the Krebs cycle.

4. Differences between *T. cruzi* and *L. tropica* were found in the following respects: response to change in ionic environment and pH, sensitivity to impurities in pyruvic acid, content of material absorbing at 240 mμ, and release of malonate inhibition by added malate.

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EXPERIMENTAL STUDIES ON VESICULAR STOMATITIS VIRUS

I. FACTORS INFLUENCING INFECTIVITY TITRATIONS IN THE CHICK EMBRYO

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The occurrence of foot and mouth disease in Mexico and Canada has stimulated research on vesicular stomatitis (VSV) and vesicular exanthema (VEV), two other virus diseases of food-producing animals already enzootic in this country. The recent explosive spread of vesicular exanthema from California to most of the United States has added a new urgency to the rapid and accurate identification of the etiological agent in vesicular disease outbreaks.

Vesicular stomatitis has long been recognized as a distinct clinical entity in veterinary medicine (Hanson, 1952), but surprisingly little attention has been given to the properties of the etiological agent itself since it was first shown to be a filterable virus in 1925 (Olitsky *et al*, 1928). Burnet and Galloway (1934) showed that VSV could be grown on the chorioallantoic membrane of 9- or 10-day-old chick embryos, described the histology of membrane lesions and pointed out that no pathognomonic changes were found. These findings were confirmed by Galloway and Elford (1934).

Sigurdsson's work (1943) is the only detailed study in the literature on the multiplication of VSV in the chick embryo. He found that the 7-day-old chick embryo was considerably more susceptible than the 10-day-old when inoculated by the chorioallantoic route. Temperatures in the range of 35 to 36 C were found more suitable for virus cultivation than 39 to 40 C.

One problem of this laboratory has concerned itself with characterization of VSV and VEV, one aspect of which has been a study of the VSV-chick embryo system for purposes of detection, assay and large scale cultivation. A preliminary survey revealed that growth was readily obtained in the 8- to 10-day-old chick embryo inoculated by the membrane, yolk and allantoic sac routes. The last route was selected for the work to be described because of the greater simplicity of inoculation and safety in collection of infective materials. Since very little information was available regarding VSV infections by this route, some of the conditions affecting infectivity titrations of VSV in the chick embryo are described in this report.

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MATERIAL AND METHODS

Strain of virus.—The Missouri isolate of the New Jersey antigenic type of VSV originally isolated from swine (Shahan et al, 1946) was used throughout this study. This virus was obtained from the Bureau of Animal Industry, where it had been subjected to numerous passages in cattle, swine and guinea pigs, the last 29 of which were made consecutively in guinea pigs. Infected foot pad coverings of the 53rd passage were the source material for the present study. A 10% foot pad suspension inoculated into the allantoic cavity of 10-day-old chick embryos killed all within 24 hours. The extra-embryonic fluids were harvested, tested for sterility in thioglycollate medium, and stored at −20 C in sealed glass ampules. As required, subsequent serial passages of chick embryo virus were made in the allantoic cavity.

Chick embryos.—Embryonated eggs used in this study were obtained from a single commercial supplier and were incubated at 37 to 38 C in a relative humidity of approximately 80%.

Infectivity titrations.—Materials to be assayed were diluted in serial 10-fold steps in 1% peptone solution and 0.1 ml volumes inoculated into the

allantoic sac of 8 to 10 chick embryos through the air sac end. Only deaths occurring within 10 to 120 hours after inoculation were included in the results. Where doubt existed about the specificity of embryo deaths, fluids from individual eggs were checked for bacteriological sterility, subpassaged in chick embryos and/or tested for specific complement-fixing activity as described below. Fifty percent lethal end points were calculated by the method of Reed and Muench (1938) and were expressed as the negative logarithm of the end point dilution, based on 0.1 ml volume.

Technique of qualitative complement fixation text.—Allantoic fluids to be tested for presence of VSV antigen were mixed with an excess of VSV guinea pig antiserum and a unit of guinea pig complement added. Two-tenth ml volumes of each reagent were used throughout. The mixtures were incubated 30 minutes at 37 C, after which sensitized cells were added and incubation continued for 10 to 15 minutes or until hemolysis was observed in the control tubes. Positive and negative controls, as well as anticomplementary controls for each test fluid, were included.

Characteristics of allantoic sac infection with vesicular stomatitis virus in the 9 to 10-day-old chick embryos

Inoculation of VSV into the allantoic cavity of 9 to 10-day-old chick embryos was usually followed by death of the embryo within 20 to 72 hours. No changes in the gross appearance which could be considered specific for this virus infection have been noted. The schematic pattern of results obtained with allantoic sac titrations of VSV is shown in table 1, summarizing 11 replicate assays of a single pool of virus. In general, there was a fairly close correlation between the concentration of virus inoculated, the associated mortality and the time of embryo death.

One rather striking feature was the comparatively narrow range of time (20 to 36 hours after inoculation) within which most of the embryos died. When large amounts of virus were inoculated (i.e., more than 100 LD_{50}'s) 80 to 100% of the embryos died before the 35th postinoculation hour. With decreased inoculums the averge hour of death increased to 60 hours, although some deaths occurred as late as 96 hours after inoculation.

TABLE 1.—*Distribution of embryo deaths after allantoic sac inoculation with graded amounts of vesicular stomatitis virus.**

Virus dilution inoculated	Embryo deaths / Total inoculated	Percent mortality	Average hour of death
10^{-1}	80/80	100	22
10^{-2}	80/80	100	25
10^{-3}	140/140	100	29
10^{-4}	112/140	80	35
10^{-5}	93/140	67	29
10^{-6}	74/140	53	40
10^{-7}	11/80	14	60

* Based on summary of 11 replicate titrations of a single virus pool in 10-day-old embryos.

In chick embryo titrations of VSV death may be considered evidence of infection, since failure of an embryo to die was closely correlated with failure to detect VSV in the extra-embryonic fluids (mixed amniotic and allantoic) either by immunologic or infectivity test. Table 2 presents results of three titrations in which the fluids of all inoculated embryos were tested for the presence of VSV antigen by a qualitative complement fixation test. Fluids for this test were harvested either shortly after death or 120 hours after inoculation from surviving embryos. A total of 274 fluids from dead embryos were tested individually; of these 239, or 88% were found to contain VSV antigen demonstrable by complement fixation. Such fluids were invariably infective on subpassage. Twenty-four of 33 (70%) fluids from dead embryos which were ***negative*** in CF tests were nevertheless infective by subpassage to other chick embryos. None of the fluids from embryos alive at 120 hours contained VSV antigen detectable by complement fixation nor were these fluids infective for chick embryos when subpassaged undiluted. These results, and those of a large number of similar smaller tests,

TABLE 2.—*Presence of complement-fixing antigen in extra-embryonic fluids of living and dead embryos inoculated with vesicular stomatitis virus.*

Virus dilution inoculated	Embryo deaths	Number of fluids tested	Fluids from *dead* embryos			Fluids from *living* embryos		
	Total inoculated		Positive CF	Negative CF	Negative CF; positive subpassage	Positive CF	Negative CF	Negative CF; positive subpassage
10^{-1}	57/57	57	50	7	7			
10^{-2}	44/44	42	39	3	3			
10^{-3}	86/86	86	75	11	9			
10^{-4}	38/45	45	36	2	1	0	7	0
10^{-5}	28/45	44	24	4	3	0	16	0
10^{-6}	13/45	44	10	3	0	0	31	0
10^{-7}	8/45	45	5	3	1	0	37	0
10^{-8}	0/15	15				0	15	0
Totals	274/382	378	239	33	24	0	106	0

established the essential identity of the 50% lethal and infective end points in chick embryo titrations of the Missouri isolate, and the validity of using embryo death as in index of specific VSV infection.

Allantoic fluid from consecutive passages of VSV yielded LD_{50} titers in the 9- to 11-day-old chick embryos which varied between 4.0 to 6.0/0.1 ml. This two-log range in titer occurred not only with different preparations but in repeated titrations of the same pool of infective fluids. Such fluctuation in titer could not be consistently related to length of storage, method of titration, or any other obvious variable. Before undertaking any quantitative study of VSV in the chick embryo, it was considered essential to learn what effect certain experimentally controllable factors might have in determining LD_{50} in this system. Some results of this analysis are presented below.

Factors influencing the LD_{50}

1. Effect of incubator temperature on infectivity assays.—The temperature of incubation routinely used for infected embryos in the early phase of this study was 37 to 38 C. It was decided to test the effect of a lower temperature in the light of Sigurdsson's (1943) observation that VSV grew better at lower (35 to 36 C) than at higher (39 to 40 C) temperatures.

For each experiment the infective fluids were thawed rapidly at 37 C, diluted to 10^{-7} and inoculated into the allantoic cavity of 10-day-old embryos, 20 embryos per dilution. Half of each group was incubated at 35 to 36 C, the other half at 37 to 38 C during the 120-hour observation period.

Results of the three comparative experiments are presented in table 3. No significant differences in time or distribution of embryo deaths nor in the final LD_{50} were found which could be attributed to the temperatures of incubation. The maximum observed differences, 0.5 log, was not considered significant. The rather large differences in titer in the three tests at either temperature were typical of the fluctuations observed in repeated titrations of a single virus pool, but were not related to temperature of incubation.

In the following paper of this series (Madin and McClain, 1956), the effect of temperature on virus multiplication rather than virus assay will be discussed.

2. Effect of age of embryo on LD_{50}.—

TABLE 3.—*Effect of incubator temperature on infectivity assay of a single infective pool of vesicular stomatitis virus (35 NJM A-12).*

Exp. no.	Virus titer	
	35 C	37 C
I	5.6	5.6
II	4.6	4.1
III	4.9	4.5

The 9- to 12-day-old chick embryos are those most commonly used for virus cultivation in the allantoic sac. This route and these embryo ages were used in this study for simplicity of inoculation and the safety with which large amounts of infective materials could be obtained. Since host age is known to determine susceptibility to many viruses (Sigel, 1952), the relationship between embryo age and LD_{50} was examined as a possible contributor to the observed fluctuations in VSV titers.

Six comparative experiments were carried out using three different virus preparations (table 4). The procedure was essentially similar for all in that virus of a given preparation was titrated in embryos of several ages. However, the experiment with the 29 NJM passage virus differed from the other five in one important respect, in that it was titrated on 8 consecutive days in progressively more mature embryos. This involved daily preparation of a new series of dilutions from a freshly thawed aliquot of the 29 NJM pool. Only in one instance was the same series of dilutions assayed in embryos of more than one age; this series was used for the titrations in 9- and 10-day-old embryos.

In five experiments with 32 and 35 NJM passage virus, embryos of the ages to be tested were available on the same day, so that in any single experiment all embryos (vertical columns II to VI, table 4) were inoculated with aliquots of the same series of dilutions. This change of procedure was designed to make the comparison somewhat more rigorous by eliminating possible differences in diluting techniques. All inoculations were completed within 20 minutes after preparation of dilutions in order to minimize possible light inactivation of the virus, such as reported by Skinner (1954).

TABLE 4.—*Effect of embryo age on LD_{50} end point of three pools of vesicular stomatitis inoculated into the allantoic cavity.*

Age of embryo used for assay (days)	Virus titer					
	29 NJM I	32 NJM II	32 NJM III	35 NJM IV	35 NJM V	35 NJM VI
7	6.0	6.1				5.3
8	4.7	4.7		5.7	5.6	
9	5.5	4.6	5.2			
10	4.5	5.0	4.3	4.5	3.6	3.7
11	4.5		2.7			
12	3.5		2.8	4.9	4.2	
13	2.0		1.5			0.9
14	1.0					
15	ND		0.0			

Results of the 6 experiments are shown in table 4. It is obvious that within a period of 7 days, the chick embryo undergoes an extreme reduction in its susceptibility to VSV introduced into the allantoic cavity. Approximately 10,000 times more virus was required to produce a lethal effect in the 13-day-old embryo than in 7-day embryos. The 14- and 15-day-old embryos were almost completely resistant to the lethal effect of VSV under these conditions, while embryos 9 to 12 days of age were intermediate in susceptibility. In general, there was a progressive increase in resistance to VSV inoculated by this route as the chick embryo matured.

The data of table 4 also illustrated very clearly some of the irregularities encountered in titrating this virus. For example, in experiment I (29 NJM) the LD_{50} titer in 12-day chick embryos was a full log lower than that in 10-day embryos, and in experiment III (32 (NJM) the difference in titer between the two was 1.5 log. In experiments IV and V, the titer in 12-day embryos was 0.4 and 0.6 log *higher*, respectively, than the titer in 10-day-old embryos. Other examples of irregularity may be seen in the comparison of the titers of 32 NJM and 35 NJM in 10-day and 12-day chick embryos. If one examined only the data in 10-day-old embryos, one might conclude that these two pools had approximately the same degree of

infectivity in the chick embryo, but comparison of the same material in 12-day-old embryos would give information that 35 NJM had almost 100-fold greater activity. This shift in LD_{50} differential cannot be attributed to minor technical details, but can only reflect some unexplained variability of the embryo host in response to this virus.

3. Comparison of allantoic sac with chorioallantoic membrane routes for determination of LD_{50}.—Another factor which is well known to influence infectivity end point determinations is the route of inoculation. Having encountered irregularities of LD_{50} values with the allantoic sac route of infection, it was pertinent to determine whether another mode of inoculation or another index of virus activity, such as membrane lesions, might provide a more satisfactory assay system.

Comparison of the two routes was made by titrating a number of virus preparations using the same series of dilutions for each comparison. Allantoic sac inoculations were made as described above; membrane inoculations were made through an artificial air-sac. The volume of inoculum was 0.1 ml for each route.

A number of typical results are summarized in table 5. The greater susceptibility of the chick embryo to VSV when inoculated onto the membrane was apparent in the consistently higher LD_{50} obtained for all ages of embryos tested. The membrane titer usually exeeded the allantoic sac titer by one log, in 7- to 8-day embryos and by 2 to 3 logs in 10- to 12-day embryos.

TABLE 5.—*Comparison of allantoic sac with chorioallantoic membrane route for infectivity titrations of Missouri strain vesicular stomatitis virus.*

Virus preparation	Age of embryo (days)	Virus titer LD_{50}		Titer difference CAM-AS*
		Allantoic titration	Membrane titration	
35 NJM A12	8	5.7	6.6	0.9
	10	4.5	7.2	2.7
	12	4.9	6.0	1.1
37 NJM A13	8	5.6	6.8	1.2
	10	3.6	6.6	3.0
	12	4.2	7.2	3.0
38 NJM A14	10	4.4	7.2	2.8
	10	4.4	7.2	2.8
	10	4.0	6.2	2.2
	10	4.9	6.6	1.7
	7	6.8	7.7	0.9
	10	4.9	7.1	2.2

* CAM—chorioallantoic membrane; AS—allantoic sac.

There appeared to be no indication that the membrane route offered any marked advantage over the allantoic sac route so far as reproducibility of lethal end points was concerned. Variation in LD_{50} of a full log may be expected when the same virus preparation is tested repeatedly in embryos of the same age whether inoculated on the membrane or in the allantoic sac.

DISCUSSION

The virus of vesicular stomatitis has a number of characteristics to recommend it to virologists of the "hothouse variety" (Hanson, 1952) interested in basic processes of viral multiplication and pathogenesis. Not only does it infect a wide range of hosts both naturally and experimentally, but it possesses neurotropic as well as dermotropic potentialities. In the chick embryo it grows extremely rapidly even with small inoculums, producing death as early as 16 hours after inoculation (as early as 10 hours with some strains) and yielding high infectivity in the membrane, embryonic tissue, and extra-embryonic fluids. While the virus has been found infective for man under field conditions as well as in the laboratory, simple precautionary measures make the virus amenable to safe handling. An additional requisite for quantitative studies is the ability to assay the virus by some accurate, reproducible technique. It is to this particular problem that the present study has been directed.

Several factors known to influence

virus titrations in chick embryos were investigated, of which embryo age and route of inoculation were found to be most important in VSV chick embryo system. There was clear evidence of increased resistance to allantoic sac infection as embryos matured, ranging from high susceptibility at 6 to 7 days and essentially complete resistance after 14 days of age. However, it was frequently noted by repeated titrations in embryos of the same chronological age that a wide range of response existed, particularly in 9- to 12-day embryos, as shown by the marked fluctuations in LD_{50} values which were observed.

There is some evidence that this variability may not be characteristic of all strains of VSV in the chick embryo. It has also been found that a higher degree of reproducibility of LD_{50} titers may be obtained by use of the more susceptible 7-day-old chick embryos. Nevertheless, the observed fact of the high variability of interaction in the chick embryo-VSV system imposes certain restrictions on conclusions which may be drawn from infectivity titrations done at different times, and limits the precision with which one can study the activities of this virus.

There are several possible explanations for the greater susceptibility to VSV when inoculated directly onto the membrane as contrasted with indirect exposure of membrane cells through the allantoic fluids. Using the data of Romanoff and Romanoff (1951), it does not seem that simple dilution of the inoculum in the allantoic fluid could account for the lesser effect. Nor is there any evidence that virus-inactivating substances accumulate in allantoic fluid as chick embryos mature. Rather than inactivation we have, in fact, observed in a small number of experiments a pronounced potentiating or at least stabilizing effect of normal allantoic fluid from 14-day-old embryos on the infectivity of VSV.

It is further conceivable that the ectodermal cells of the membrane respond quite differently to VSV than the endodermal cells of the membrane which line the cavity. Such a difference in response was reported by Isaacs and Fulton (1953) with an influenza virus. We have found no evidence that virus growth, as measured by infectivity titer, is any different for the two routes of inoculation. The virus content of membranes and fluids when infection does occur are essentially the same regardless of the route of infection.

In spite of the greater sensitivity of the embryo to infection by the membrane route, this mode of inoculation does not appear to have a marked advantage over that of the allantoic sac for routine assay purposes. The range of experimental error for both routes is approximately the same, that is, about one log. Considering the greater ease of allantoic sac inoculations, this route represents the one of choice for infectivity assays. Use of the 7-day chick embryo in such titrations is advised for their greater sensitivity to infection and more uniform response.

SUMMARY

1. A study of certain characteristics of allantoic sac infection of the chick embryo with VSV indicates:

a. Death of 80 to 100% of embryos inoculated with VSV occurs within 35 hours after inoculation and is not accompanied by pathognomonic changes.

b. Death of the embryo in VSV titrations may be considered evidence of infection as indicated by immunologic and infectivity tests.

2. Certain factors influencing infectivity titrations by the allantoic sac route were investigated with the following results:

a. Varying the temperature of incubation of eggs infected with VSV has little or no effect on the final LD_{50} titer.

b. The susceptibility of the chick embryo to VSV decreases markedly with increasing age.

3. The chick embryo is more susceptible when inoculated by the chorioallantoic membrane route than by the allantoic sac route.

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AMINO ACID REQUIREMENTS OF ORAL LACTOBACILLI

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Previous communications from this laboratory have dealt with the vitamin requirements of a collection of oral lactobacilli.[1-3] As part of a continued investigation of the nutritive requirements of these organisms the amino acid needs also have been studied and the results are presented in this report.

Although the amino acid needs of some lactobacilli, particularly the strains suggested for use in microbiological assays, have been studied in considerable detail, the requirements of oral lactobacilli have been relatively neglected. In a general way, however, it is usually supposed that the amino acid requirements of lactobacilli from the mouth are probably as complex as those of lactobacilli used for assays or those from dairy and fermentation sources. Earlier attempts to cultivate oral lactobacilli in simple synthetic mediums uniformly met with failure. During the studies on vitamin requirements it became apparent that protein hydrolyzates were necessary to supply a number of amino acids and that it was advisable to supplement acid hydrolyzates with tryptophane and cystine. Aside from this recognition of the importance of a number of amino acids for oral lactobacilli, relatively little information of specific needs has been available; Weisberger and Johnson[4] noted that tyrptophane was needed by an oral lactobacillus, and Dreizen and associates[5] determined the needs of an *L. acidophilus* strain.

In contrast, the amino acid needs of lactobacilli from other sources have been studied in detail. Typical of these reports are those dealing with *L. arabinosus* 17–5 (8014) by Kuiken, Norman, Lyman and Hale[6] and by Hegsted,[7] with *L. pentosus* 124–2 (8041) by Krueger and Peterson,[8] and with *L. casei* by Hutchings and Peterson.[9] The amino acid needs of the foregoing species along with *L. fermenti* 36 (9338) and a number of others (though apparently none of definitely oral origin) were also studied by Dunn and associates.[10] The needs of two strains of *L. leichmannii* were determined by Schweigert, Guthneck, and Scheid.[11] A detailed review of the use of various microorganisms—lactic acid producing and others—for amino acid assay was published by Dunn,[12]

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and more recently Hendlin[13] discussed some of the problems connected with use of lactic acid bacteria in microbiological assays.

It has become clear that the needs of the foregoing lactobacilli are complex; often a number of amino acids are necessary to secure satisfactory growth. When the amino acid needs have been studied by the precedure of omitting singly each amino acid from a mixture of 18 or 20 amino acids, often 8, 10 or more amino acids have been found necessary for growth. However, this procedure has not always yielded the whole story of amino acid needs. Thus, Hegsted[7] pointed out that although 10 amino acids were determined as essential for *L. arabinosus* 17–5 by this procedure, when the 10 were supplied in the absence of the others the mixture was not adequate for growth. For "good growth" 13 amino acids, out of a total of 19 studied, were needed. Similar findings were reported by Hutchings and Peterson[8] for *L. casei* and by Krueger and Peterson[8] for *L. pentosus* 124–2; 16 or 17 amino acids were necessary for satisfactory growth of these lactobacilli.

The amino acids often found essential for the foregoing lactobacilli include valine, isoleucine, leucine, arginine, glutamic acid, cystine or cysteine, tyrosine, phenylalanine, and tryptophane. But for satisfactory growth usually several, or at times all, of the following have been used in addition to the others: alanine, serine, threonine, lysine, aspartic acid, methionine, histidine, and proline. The needs have differed with the different species and with different strains within the species. The matter has been complicated further by erroneous nomenclature of some of the laboratory stock cultures of these organisms.

13. Hendlin, D., 1952, Bact. Rev. 16: 241–246.

There is a growing realization that lactobacilli isolated from the oral cavity do not constitute a single species or even a subgroup apart from other lactobacilli, but that most of them possess the characteristics of species encountered elsewhere in nature. Thus, the lactobacilli most commonly encountered by Rogosa and associates[14] in an extensive study of 500 oral isolates were similar or identical with *L. casei*, *L. plantarum*, *L. acidophilus*, *L. arabinosus*, *L. fermenti*, *L. buchneri*, and *L. brevis*. Only relatively few isolates possessed other characteristics or were unidentifiable. Similarly, most of the 200 or more oral lactobacilli studied by Tilden and Svec[15] could be fitted satisfactorily into the foregoing species. Both groups of investigators agreed that *L. casei* was the most frequently encountered of the homofermentative type and *L. fermenti* of the heterofermentative type.

In view of these results it might perhaps be anticipated that the amino acid needs of the oral lactobacilli are much the same as those of other lactobacilli which have been studied in more detail. This, however, is not definitely known, and to gain more information concerning needs of oral lactobacilli this study was undertaken. If the amino acid requirements should be found to be essentially similar to those of other homofermentative and heterofermentative lactobacilli (with due allowance for differences at times seen in individual strains), then this would be added evidence for the merging of the oral lactobacilli with other species now recognized. On the other hand, if the amino acid requirements of oral lactobacilli should differ in general from other

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lactobacilli, then the difference might be significant from the standpoint of classification, as well as a contribution to their nutritive requirements.

METHODS

Basal medium.—The basal medium contained 18 amino acids as well as purines and pyrimidines, vitamins, salts, acetate, and glucose in the following amounts per 1000 ml of medium: 400 mg of each of DL-alanine, L-aspartic acid, L-glutamic acid; 200 mg of each of DL-isoleucine, DL-leucine, DL-methionine, DL-phenylalanine, DL-serine, DL-threonine, and DL-valine; 100 mg of each of L-arginine monohydrochloride, glycine, L-histidine, L-lysine dihydrochloride, L-proline, L-tryptophane, and L-tyrosine; 50 mg of cysteine monohydrochloride; 10 mg of each of adenine sulfate, guanine hydrochloride, xanthine, thymine, and uracil; 10 μg biotin, 1000 μg nicotinic acid, 500 μg calcium pantothenate, 100 μg pteroylglutamic acid, 100 μg para-aminobenzoic acid, 300 μg pyridoxal hydrochloride, 200 μg riboflavin, and when heterofermentative lactobacilli were used 500 μg thiamine hydrochloride. The inorganic salts were 3 g K_2HPO_4, 3 g NH_4Cl, 200 mg $MgSO_4 \cdot 7H_2O$, and 10 mg of each of NaCl, $FeSO_4 \cdot 7H_2O$, and $MnCl_2 \cdot 4H_2O$. Also 10 g sodium acetate and 15 g glucose were used.

The medium was adjusted to pH 6.8 before autoclaving and was usually about 6.4 to 6.5 after autoclaving. The ingredients for one liter were dissolved in either 900 or 1000 ml of glass redistilled water, placed in 9 or 10 ml amounts in tubes previously standardized for turbidity determination in the Lumetron. After addition of any sterile solutions just before inoculation the volume per tube was approximately 10 ml.

Thiamine and cysteine were added separately from filtered solutions just before inoculation and after the medium had been tubed and autoclaved. Other compounds were added to the medium directly and autoclaved in the medium. When *L. acidophilus* was used the following compounds were added from previously filtered solutions to each 10 ml tube of medium: Tween 80, 1 mg; ascorbic acid, 3 mg; pantethine, 2 μg, thiamine hydrochloride, 2 μg, and vitamin B_{12}, 0.1 μg.

Cultures.—Thirty-three strains of lactobacilli were used for the tests. This number included 28 oral strains and 5 named species from other sources, American Type Culture Collection or other laboratories, which were used for comparative purposes. Twenty-six of these cultures were homofermentative, 7 were heterofermentative. Within each of these two categories were representatives of different species or varieties found commonly in the mouth. In a separate study of the characteristics based on the usual fermentation and other tests commonly applied to this group of organisms, the 28 oral strains were classified as follows: 9 were *L. casei*, 4 were considered as lactose-negative *L. casei*, 3 were *L. plantarum*, 4 were *L. acidophilus* 5 were *L. fermenti*, 1 was *L. brevis* and 2 were not assigned.

Of the 9 *L. casei* cultures 3 failed to ferment rhamnose, the other 6 fermented this sugar. The 4 cultures termed lactose-negative *L. casei* resembled *L. casei* in most characteristics but did not ferment lactose; their characteristics were quite different from those of the lactose-negative *L. delbrueckii* and *L. leichmannii*. Similar *L. casei*-like cultures were noted by Rogosa and associates[14] (their variety *alactosus*) and by Tilden and Svec[15] in their studies of the oral flora. The 5 known species from sources other than the mouth were: *L. casei* 7469, another strain identified as *L. casei*, *L. arabinosus* 17-5 (or ATCC 8014), *L. pentosus*, and *L. fermenti* 9338. *L. arabinosus* and *L. pentosus* are at times referred to as *L. plantarum*.

For inoculation of tests, 0.001 ml, secured by dilution, of 24-hour cultures was used for each tube.

Growth and acid production.—Incubation was at 37 C. Growth response of the lactobacilli was followed by measurements of turbidity in a Lumetron colorimeter with a 650 mμ filter. pH determinations were made also in many instances, using a Leeds and Northrup potentiometer.

RESULTS

In the complete medium of 18 amino acids all lactobacilli except *L. acidophilus* produced heavy final growth and most of them grew readily. The 4 *L. acidophilus* cultures did not produce as luxuriant growth as the others but nevertheless grew sufficiently well so that the effect of omissions of amino acids was readily apparent. With these results for comparison, the essentiality of various amino acids was determined by omitting them singly from the basal medium and noting the growth response of the organisms.

The results of numerous tests are not given in detail, but some typical results

showing the effect of omission of each of two amino acids are given in table 1. Here it is seen that when glutamic acid was omitted, with all other amino acids supplied, all strains failed to grow. Glutamic acid is required for growth. When threonine was the only amino acid omitted all homofermentative lactobacilli, except one strain of *L. acidophilus*, grew as well as in the complete medium. Threonine was not required by these organisms. In contrast, 4 of the 5 strains of *L. fermenti* (heterofermentative) required threonine and the remaining one was stimulated by it. An oral strain of *L. brevis* and a type culture collection strain (9338) of *L. fermenti* also required threonine for growth (not shown in table 1). This difference in requirement for threonine shown by homofermentative and heterofermentative cultures was one of the few differences between these fermentative subgroups of lactobacilli encountered in the study of amino acid requirements.

TABLE 1.—*Effect on growth of omission of either glutamic acid or threonine.*

Species and strain No.	Complete amino acid medium	Lacking only glutamic acid	Lacking only threonine
	2 or 3 days	7 days	2 or 3 days
L. casei			
3	71*	2*	73*
45	85	0	81
46	79	1	74
1003	69	0	80
Lactose-negative *L. casei*			
4	81	1	79
1009	79	0	83
L. plantarum			
1019	88	1	87
1020	89	2	90
1023	87	2	87
L. acidophilus†			
1025	36	7	35
1026	48	8	14
			5 to 10 days
L. fermenti			
6	88	0	0
41	77	0	80‡
55	89	0	0
78	85	0	0
1004	83	1	0

* The figures in the table represent comparative growth as shown by turbidity. Turbidity determinations were made with a Lumetron colorimeter using a 650 mμ filter. The readings of percent light transmission were subtracted from 100 to show increasing turbidities by higher figures: 0 = clear tube; 65–85 = heavy turbidity, near maximal or maximal growth.

† The *L. acidophilus* cultures grew slowly and the readings shown are those obtained at 4 or 5 days, rather than 2 or 3 days as used for the other cultures.

‡ Growth of culture 41 was slow in the absence of threonine, requiring usually 8 to 10 days to attain the growth shown here.

In a similar way, the needs of the 28 strains of oral lactobacilli and the 5 strains from other sources were tested by omission of each of the 18 amino acids from the mixture. Thus the amino acid requirements, as determined by these tests, are those which become apparent when all amino acids except one were supplied, some of them supplied presumably in considerable excess over the actual amounts needed. The medium contained all other compounds known to be needed for growth of the strains used. A summary of the amino acid requirements as determined by many hundred individual tests, including repetitions to check results, is given in table 2.

On examination of these results several conclusions seem justified:

1. Irrespective of individual differences, the oral lactobacilli as a whole are rather exacting in their amino acid requirements. Most of them have an absolute requirement for at least 8 or more amino acids. Several additional amino acids, though not an absolute requirement, serve to stimulate growth, in some instances quite markedly.

2. Certain amino acids are required by all or almost all cultures, irrespective of strain or species distinctions: arginine, cysteine (or cystine), glutamic acid, leucine, phenylalanine, tryptophane, tyrosine, and valine. Isoleucine is markedly stimulatory for most cultures and required by some.

3. Within each species the individual strains show some differences, as might be expected. Thus, some oral *L. casei* strains were stimulated by aspartic acid (in the presence of biotin) but others

TABLE 2.—*Summary of amino acid needs of oral lactobacilli.*

	L. casei 9 strains	*L. casei* lactose negative, 4 strains	*L. plantarum* 3 strains	*L. acidophilus* 4 strains	*L. fermenti* 5 strains
DL-alanine*	O	O	O	S	S†
L-arginine	R	R	S	R	R
L-aspartic acid*	S‡	S‡	O	S	S‡
L-cysteine	R§	R	R, S	R	R§
L-glutamic acid	R	R	R	R	R
Glycine	O, S	O, S	O	O, S	O, S
L-histidine	O‖	O	O‖	R	O, R
DL-isoleucine	S	S	S	S†	S, R
DL-leucine	R	R	R	R	R
L-lysine	O†	O	O	R§	O
DL-methionine	O	O	O	S, R, O	O
DL-phenylalanine	R	R	S, R	R	R
L-proline	O	O	O	R	O
DL-serine	S	S	O	R, S, O	O
DL-threonine	O	O	O	R, O	R§
L-tryptophane	R	R	R, S	R	R
L-tyrosine	R	R	O, S, R	R	R
DL-valine	R	R	R	R	R

R = required for growth, O = not required, S = stimulatory. The use of more than one symbol opposite an amino acid signifies the result was about equally divided with respect to the number of cultures tested.

* Tests of alanine and aspartic acid were made in the presence of pyridoxal and biotin. In the absence of either of the vitamins, the respective amino acids are more important.

† One exception, required by one strain.

‡ Aspartic acid stimulated growth of many of the cultures; a few grew equally well without aspartic acid.

§ Stimulatory rather than required by one or two cultures.

‖ Stimulated growth of an occasional strain though not required.

were not. A few strains of this species were stimulated by histidine, one required lysine for growth while 8 other strains did not. Similar differences were encountered at times among the individual strains of other species.

4. In spite of individual strain differences, a few species distinctions are apparent in a general way. *L. casei* is slightly more exacting with respect to the amino acids needed for optimal growth than is *L. plantarum*. *L. acidophilus* is the most exacting; indeed, some strains of *L. acidophilus* either require or are stimulated by practically all of the 18 amino acids used in the study.

5. The *L. fermenti* cultures (heterofermentative) exhibit a few distinctions from the commoner homofermentative species such as *L. casei* and *L. plantarum*. *L. fermenti* usually requires threonine and is stimulated by DL-alanine in the presence of pyridoxal, but it is not stimulated by serine, as is *L. casei*. Similar results were secured with *L. fermenti* ATCC 9338 and an *L. brevis* culture of oral origin, which are not shown in table 2. *L. brevis* closely resembles *L. fermenti* in many characteristics.

The dispensability of DL-alanine was tested in the presence of pyridoxal. Under such condition omission of DL-alanine had little or no effect on growth of *L. casei* and *L. plantarum*. In additional tests involving the use of DL-alanine in the absence of pyridoxal (not shown in table 2) it was found that many *L. casei* cultures grew poorly. For these organisms DL-alanine did not substitute satisfactorily for pyridoxal in the amino acid medium used in these tests. These *L. casei* strains may be similar to *L. casei* 7469 studied by Snell and associates,[16–18] which required, in the absence of vitamin B_6, not only D-alanine but certain peptides containing L-alanine, rather than free L-alanine.

Among the amino acids which exerted a stimulatory effect on some cultures was glycine. The effect was not pronounced, but was definite and was seen on repeated tests. This effect may have been due to the formation of glucosyl-

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glycine on autoclaving the medium.[19]

Certain other results not included in table 2 may be mentioned briefly. The two oral strains, previously not assigned definitely to any species, were found to have amino acid needs quite similar to those of *L. casei*, which they somewhat resembled in other respects. *L. casei* ATCC 7469 showed essentially the same amino acid requirements as the oral *L. casei* strains of table 2. Known-type *L. arabinosus* and *L. pentosus* cultures, which are closely allied or identical with *L. plantarum*, exhibited requirements essentially similar to the oral *L. plantarum* cultures of table 2. Thus there seemed to be no real differences between the oral lactobacilli of each species and the known-type lactobacilli from other sources, on the basis of the limited number of cultures of each species included in this study. Additional strains were not included since the purpose of the study was to obtain an over-all picture of requirements of the commoner oral lactobacilli, rather than the study of a larger number of strains of one or a few species.

Effect of nonessential amino acids.—In the foregoing experiments the absence of certain amino acids, when omitted one at a time, exerted little or no apparent effect upon either the speed or final amount of growth when the remaining 17 amino acids were supplied. These amino acids are usually designated as not required or nonessential. Yet when several amino acids of this category are all omitted at the same time, there is often a definite effect upon growth, even though all essential amino acids (as determined on the basis of single omissions) are supplied. Growth under such conditions is slower and not as luxuriant; at times it may fail to appear, especially in successive transfers.

In the presence of 10 or 11 amino acids, including all essential ones, only a few *L. casei* and some *L. plantarum* cultures grew well. Growth of the others was delayed or was submaximal in amount, even though sufficient quantities of these amino acids were supplied. Growth of all *L. fermenti* cultures was poor in the presence of 13 amino acids. When the omitted amino acids were supplied, either singly or in groups of 2 or 3, they exerted a cumulative effect in improving growth. Usually, from 13 to 16 amino acids were necessary for maximal growth and acid production of *L. casei*, lactose-negative *L. casei*, the remainder of the *L. plantarum* cultures, and *L. fermenti*. The exact needs varied somewhat with the individual cultures. *L. acidophilus* needed practically all of the 18 amino acids for satisfactory growth; only one strain of this species grew slowly when 14 amino acids were supplied. The results with the oral lactobacilli as a group are similar to those noted earlier for the several strains of lactobacilli suggested for assay purposes.[7–9]

Amounts of amino acids.—The next phase of the investigation was to determine for some of the amino acids the approximate amounts necessary for full growth and acid production. The tests were made by supplying graded amounts of one amino acid in the usual chemically defined medium containing the customary amounts of each of the other 17 amino acids. Several strains of both homofermetative and heterofermentative subgroups were chosen for the tests.

The result of an experiment with valine is given in table 3. Of the 7 strains of lactobacilli tested in the presence of varying quantitities of DL-valine, 5 were homofermentative and 2

19. Rogers, D., and King, T. E. and Cheldelin, V. H. 1953 Proc. Soc. Exper. Biol. & Med. 82: 140–144.

TABLE 3.—*Effect of graded amounts of DL-valine on growth and acid production.*

DL-valine μg/ml	Turbidity							pH						
	Lactobacilli							Lactobacilli						
	4	45	46	1009	ATCC 7469	1004*	ATCC* 9338	4	45	46	1009	ATCC 7469	1004*	ATCC* 9338
0	1	1	2	3	3	1	1	6.2	6.3	6.2	6.2	6.1	6.3	6.3
1	11	11	11	7	12	1	2	5.2	5.2	5.4	5.5	5.2	6.3	6.3
4	29	27	37	27	32	4	31	4.6	4.6	4.5	4.7	4.4	6.2	5.2
10	48	45	48	46	47	22	42	4.2	4.3	4.2	4.3	4.2	5.5	4.9
20	70	49	65	69	65	53	67	3.9	4.0	3.9	4.0	3.9	4.7	4.4
40	78	75	79	77	78	69	84	3.7	3.8	3.8	3.8	3.7	4.4	4.2
100	84	85	81	86	83	70	87	3.7	3.7	3.7	3.8	3.7	4.5	4.2

Turbidity and pH determinations were made at 3 days at 37 C. Turbidity figures are stated as in table 1.
ATCC 7469 is *L. casei*.
* Heterofermentative cultures, *L. fermenti*.

heterofermentative; 2 ATCC strains were included for comparison with the oral strains. Maximal growth and acid production were attained in the presence of either 40 or 100 μg per ml of DL-valine. An increase in the amount to 200 μg per ml (not shown in the table) did not result in heavier growth or lower pH values. Forty μg per ml supported about the same amount of growth as 100 μg in the case of 3 cultures and near maximal growth of the other 4 cultures. Since the results with 40 μg were so close to those of 100 μg, intermediate amounts were not used. With 20 μg and smaller amounts of valine, growth and acid production were suboptimum. A composite result for all 7 strains may be stated as 40 to 100 μg per ml of DL-valine needed for maximal growth under the conditions of these tests.

In a similar manner tests with graded amounts of other essential amino acids were made. In general the same strains of lactobacilli were used, though in some cases not all 7 of them. Composite results of these tests are listed in table 4. It seemed advisable to state the amounts in rather general terms since results with the different strains often varied somewhat. In each case where a range is given, such as 40 to 100 μg per ml, the smaller figure supported nearly maximal growth and acid production, in many cases only a trifle less than that given by the higher figure. It is evident that the amounts of different amino acids needed for maximal growth differ considerably. On the one hand, 5 μg per ml of tryptophane satisfied the need of most cultures; at the other extreme, 100 μg or more of L-glutamic acid was needed for maximal growth. The latter result, it may be noted, was secured in the presence of 400 μg per ml of aspartic acid. It must be emphasized that these results apply only to the synthetic medium used. Modifications in the medium might well produce changes

TABLE 4.—*Amounts of amino acids needed for maximal growth and acid production.*

	Homofermentative* μg per ml		Heterofermentative* μg per ml	
DL-alanine†	100	(1)	100	(4)
DL-alanine			40–100	(3)
DL-valine	40–100	(6)	40–100	(3)
DL-leucine	40	(6)	40	(2)
			40–100	(1)
DL-isoleucine	20– 40	(3)	10	(1)
			20– 40	(2)
DL-threonine			20– 40	(4)
			40–100	(2)
L-arginine hydrochloride	20	(2)	20– 40	(1)
	20– 40	(3)	40–100	(1)
L-glutamic acid‡	100	(4)	100	(3)
	100–200	(1)		
L-cysteine·HCL	40–100	(5)	40	(2)
			40–100	(1)
L-tyrosine	10– 20	(4)	10– 20	(3)
DL-phenylalanine	20– 40	(3)	20– 40	(1)
	40	(3)	40	(2)
L-tryptophane	5	(4)	5	(3)
	5– 10	(2)		

The figures in parentheses show the total number of strains tested in each case.
* Homofermentative cultures used for these tests included representative strains of *L. casei*, lactose-negative *L. casei*, and *L. plantarum*. The heterofermentative cultures were *L. fermenti*.
† Pyridoxal was omitted from the basal medium in this case.
‡ In the presence of 400 μg per ml of L-aspartic acid.

leading to a somewhat greater or lesser requirement.

The response to tryptophane was studied by making a sufficient number of readings of turbidity in relation to time to construct growth curves of two cultures in the presence of varying amounts of this amino acid. The result with one of the cultures is shown in figure 1; the result with the other was similar. The amount of L-tryptophane needed is only about 20 to 50 times that of the vitamin nicotinic acid. These results were secured in the presence of 1.0 μg. per ml of nicotinic acid. All of the lactobacilli which we have tested require both tryptophane and nicotinic acid.

pH.—Throughout the investigation numerous determinations of pH were made along with the turbidity readings, but the results have not been stated in detail. In the complete amino acid medium *L. casei*, lactose-negative *L. casei*, and *L. plantarum*, all of which produced heavy final growth, lowered the pH of the medium from the original 6.4 or 6.5 (after autoclaving) to 3.7 or 3.8. *L. acidophilus*, which did not grow as profusely, lowered the pH

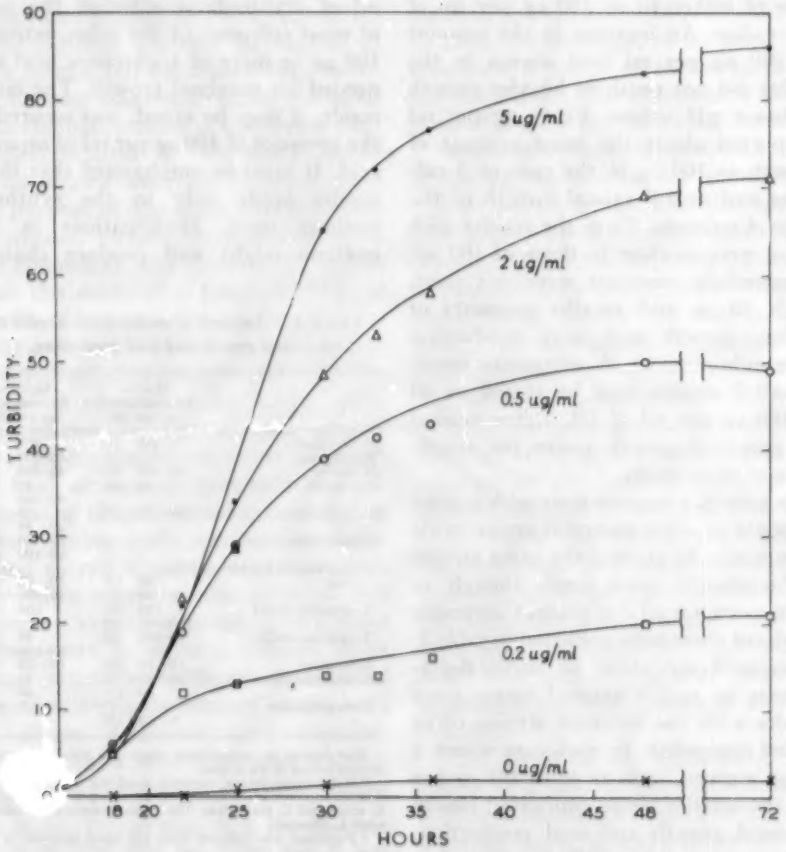

FIG. 1.—Growth response of an oral *L. casei* to different amounts of tryptophane.

usually to 4.1 to 4.6. The heterofermentative *L. fermenti* and *L. brevis* changed the pH to 4.2 to 4.4; turbidity was usually equal to that of *L. casei* and *L. plantarum*.

In those cases where the omission of a single amino acid resulted in no decrease in the amount of growth, the pH values were comparable to those produced by the cultures in the complete medium. Thus, in a medium lacking only lysine the pH values after growth of the lactobacilli were the same as in the complete medium wherever the turbidity was comparable. When the lactobacilli failed to grow or grew only sparsely, as in the absence of glutamic acid (table 1), the pH changed but little from that of the uninoculated medium. Similar results were secured throughout the tests with other amino acids.

In instances where more moderate growth or poor growth occurred, as in the presence of 11 to 13 amino acids, the pH values did not reach as low a point as in the complete medium. Lactobacilli which attained final turbidities of about 30 to 50, for example, reached pH levels of 4.6 to 4.2 for homofermentative cultures and about 5.2 to 4.7 in the case of the heterofermentative cultures. Suboptimum amounts of an essential amino acid also served to limit changes in pH. Some examples have been given in table 3. Throughout the work no instances were observed in which the drop in pH did not bear a general relation to the amount of growth as shown by turbidity.

DISCUSSION

It has become increasingly clear that the nutritive needs of bacteria are influenced by the environment. Consequently the findings with respect to amino acid requirements of lactobacilli relate to specific conditions of growth and the conclusions are not necessarily valid in other circumstances. Of the various changes which may affect the nutritive needs, it is now recognized that the presence of certain vitamins modifies the amino acid requirement. For example, Lyman and associates[20] found that in the presence of vitamin B_6 a strain of *L. arabinosus* did not require threonine, lysine, or alanine; without vitamin B_6 each of these amino acids was essential. The presence of carbon dioxide also affected the amino acid needs. Holden and Snell[17] showed that when vitamin B_6 is not available D-alanine must be supplied for *L. casei*, but when this vitamin is present the organism forms D-alanine. In general, a more complete assortment of amino acids is needed for satisfactory growth when vitamin B_6 is absent. A relationship between amino acids and pteroylglutamic acid has been noted; Harry[21] found that unrestricted growth of *L. casei* 7469 occurred in mediums without pteroylglutamic acid if certain combinations of L-glutamic acid and DL-alanine were present. That is, in the absence of the vitamin, the two amino acids assumed greater importance. An interrelation between carbon dioxide, temperature of incubation, and the need for phenylalanine, tyrosine, or aspartic acid by *L. arabinosus* was reported by Borek and Waelsch.[22]

Antagonistic effects between two or more amino acids also play a part in determining the requirement. Thus, for certain lactobacilli and other lactic acid bacteria threonine exerted an antagonistic effect on the utilization of serine;[23]

20. Lyman, C. M., Moseley, O., Wood, S., Butler, B. and Hale, F. 1947, J. Biol. Chem. **167**: 177–187.
21. Harry, E. G. 1951, Biochem. J. **49**: 5–10.
22. Borek, E. and Waelsch, H. 1951, J. Biol. Chem. **190**: 191–196.
23. Meinke, W. W., Holland, B. R. and Sample, M. B. 1948, J. Biol. Chem. **173**: 535–545.

leucine and valine in moderate amounts interfered with the utilization of isoleucine;[24] and high concentrations of aspartic acid and ethionine strongly inhibited utilization, respectively, of glutamic acid and methionine.[25] In such cases poor growth or no growth results from the use of certain combinations and proportions of amino acids and this antagonistic effect complicates the determination of amino acid requirements.

In presenting the amino acid needs of the lactobacilli studied in this investigation it is emphasized that the results apply only to the conditions used. No attempt has been made in this study to investigate the numerous interacting variables with all of the cultures, though certain relationships became evident which seem worthy of further study. The primary object at this time has been to compare in a general way the needs of a number of oral lactobacilli under a given set of conditions.

A comparison was made of several ATCC or other identified lactobacilli with lactobacilli of oral origin. These results were not included in the tables, but a striking similarity was apparent in the tests. The amino acid requirements of two *L. casei* strains were the same as those of the oral *L. casei* and lactose-negative *L. casei*-like cultures shown in table 2. Also, the needs of *L. plantarum* ATCC 8014 (also known as *L. arabinosus* 17–5) and a previously identified *L. pentosus* strain were almost identical with the oral *L. plantarum*. For all *L. plantarum* cultures arginine was stimulatory rather than a definite requirement, and serine did not stimulate growth as it did in the case of *L. casei*. *L. plantarum* 8014 did not need cysteine (in the presence of methionine) and *L. pentosus* did not need phenylalanine or tyrosine. The results with *L. fermenti* 9338 and the 5 oral cultures of the same species were similar for the most part; for 9338, arginine and threonine were stimulatory rather than a definite requirement, cysteine was not needed, but proline, in contrast to the 5 oral cultures, was a definite requirement for growth.

A comparison of our findings with previously published results for other lactobacilli can be made in some cases. The amino acid needs of the several lactobacilli used for assay purposes have been studied in detail by several groups of workers. For *L. casei* Hutchings and Peterson[9] found that arginine, aspartic acid, cystine or cysteine, glutamic acid, leucine, phenylalanine, serine, tryptophane, tyrosine, and valine were essential, while alanine, histidine, isoleucine, lysine, methionine, and threonine stimulated growth. Dunn and associates[10] studied *L. casei* 7469; the amino acids found essential were practically the same as those listed by Hutchings and Peterson,[9] with the exception that isoleucine was reported as required rather than stimulatory. A comparison of these results with the 9 strains of oral *L. casei* and the 4 lactose-negative *L. casei* given in our table 2 shows that the requirements are very similar indeed.

The amino acid requirements of *L. arabinosus* 17–5 (i.e., *L. plantarum* ATCC 8014) were studied by Hegsted[7] and by Dunn and associates.[10] The former listed arginine, cystine or cysteine, glutamic acid, isoleucine, leucine, methionine, phenylalanine, tryptophane, tyrosine, and valine as essential; several others, notably aspartic acid, lysine, and threonine were stimulatory. The 3 *L. plantarum* strains of oral origin

24. Brickson, W. L., Henderson, L. M., Solhjell, I. and Elvehjem, C. A. 1948, J. Biol. Chem. **176**: 517–528.

25. Camien, M. N. and Dunn, M. S. 1949, J. Biol. Chem. **179**: 935; 1950, J. Biol. Chem. **184**: 283.

used in the present study are less exacting in certain of their needs, particularly with respect to the methionine requirement and the lack of appreciable stimulation by lysine and threonine (table 2). Costilow and Fabian[26] studied the needs of 4 *L. plantarum* strains from cucumber fermentation; cystine, glutamic acid, isoleucine, leucine, threonine, tryptophane, and valine were required by all, while alanine, arginine, phenylalanine, and tyrosine were either stimulatory or essential for one or more of the cultures. Our 3 oral strains differed from the foregoing only in that alanine and threonine were nieither required nor appreciably stimulatory. The test for alanine, it will be recalled, was made in the presence of an ample supply of pyridoxal. In spite of a few differences, which may represent largely minor differences between individual strains, there is an over-all similarity in the amino acid needs of the oral cultures, those from cucumber fermentations and from the type culture collection.

The similarity of needs is true also of the *L. fermenti* cultures, though again some of the details differ. Strain 36 (or ATCC 9338) and *L. gayonii* ATCC 8289 (or *L. fermenti* 8289) were studied by Dunn and associates.[10] Arginine, glutamic acid, histidine, isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine, tryptophane, and valine were found essential for both of these organisms. The 5 oral *L. fermenti* and one closely-related *L. brevis* of our study, although exhibiting a number of differences between individul strains, did not require methionine in the presence of cysteine. They required cysteine or were stimulated by it.

Only in the case of *L. acidophilus* was there a distinct difference between our findings and those of others. Little information of the amino acid needs of this species has appeared, probably because this organism is one of the more fastidious of the lactobacilli and it has not been possible until recently to cultivate it is strictly chemically defined mediums. Also, there has been some confusion regarding the characteristics of this species, or just what should be considered as *L. acidophilus*. Dreizen, Mosny, Gilley, and Spies[5] found that growth of an oral *L. acidophilus* was completely inhibited when any one of 6 amino acids—cystine, glutamic acid, methionine, phenylalanine, serine, or valine—was omitted, i.e., these amino acids were essential. A number of other amino acids were classed as stimulatory since acid production was markedly reduced in their absence. These were arginine, leucine, isoleucine, proline, tyrosine, or tryptophane. The absence of alanine or threonine also reduced acid production, though not so markedly. The total number of amino acids (14) needed for maximal growth of their culture approached the number needed by our 4 strains. In general, these amino acids were the same, but in our study 12 to 14 amino acids were found to be essential rather than stimulatory, in the sense that their omission resulted in failure to grow.

26. Costilow, R. N. and Fabian, F. W. 1954, Food Res. 19: 269–271.

SUMMARY

1. The amino acid requirements of 28 strains of oral lactobacilli, representing species occurring commonly in the mouth, were determined by omission of each of the amino acids singly from a mixture of 18 amino acids. The requirements are complex; usually at least 8 to 10 amino acids, and often more, are definitely required. Other amino acids stimulate growth, in some instances quite markedly. The results have been summarized in table 2.

2. In spite of some differences in individual strains, a few species distinctions emerge in an over-all way. *L. casei* is slightly more exacting in the amino acids needed for optimal growth than is *L. plantarum*. *L. acidophilus* is more exacting than either *L. casei* or *L. plantarum*. The heterofermentative *L. fermenti*, in contrast to homofermentative *L. casei* and *L. plantarum*, usually requires threonine and is stimulated by DL-alanine in the presence of pyridoxal, but it is not stimulated by serine, as is *L. casei*.

3. Amino acid requirements of lactobacilli from the mouth are quite similar to those of the same species of ATCC or known cultures from other sources. No distinctions were observed which would would tend to separate the oral strains from lactobacilli from other sources.

4. Certain amino acids were essential for growth of most lactobacilli, irrespective of species or strain differences: arginine, cysteine (or cystine), glutamic acid, leucine, phenylalanine, tryptophane, tyrosine, and valine. Isoleucine was markedly stimulatory for all cultures and a definite requirement for some.

5. Amino acids other than those classed as essential on the basis of single omission tests play an important part in growth of lactobacilli. With a supply of 10 to 13 amino acids, including all those determined as essential, growth of *L. planarum*, *L. casei*, and *L. fermenti* is often sparse or slow. For many strains of these species 13 to 16 amino acids must be supplied for prompt and maximal growth.

6. The approximate amounts of each important amino acid necessary for maximal growth and acid production were determined by supplying graded amounts of one amino acid in the presence of an ample supply of the other 17 amino acids. The results differed considerably, from 5 μg per ml of tryptophane to 100 μg or more of glutamic acid.

STUDIES IN EXPERIMENTAL MENINGITIS IN RHESUS MONKEYS

II. THE IMPORTANCE OF STREPTODORNASE IN THE ENZYMATIC DIGESTION IN VITRO OF EXPERIMENTALLY PRODUCED MENINGEAL EXUDATE

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Streptokinase (SK) and streptodornase (SD) have been used to cause rapid lysis of purulent exudates in a variety of clinical infections.[1] SK is an enzyme activator, or kinase, which activates a pro-enzyme plasminogen, present in human serum, to form a fibrinolytic enzyme called plasmin. SD is a desoxyribonuclease which depolymerizes extracellular desoxyribonucleic acid (DNA). The solid noncellular components of purulent exudates have been shown to consist largely of fibrin and extracellular desoxyribonucleoprotein.[1,2]

The purpose of the experiments reported in the present paper was to determine the action, in vitro, of SD, SK and plasmin on exudate scraped from the meninges of experimentally infected monkeys. A purulent meningitis was produced in rhesus monkeys by the intracisternal injection of *Pseudomonas aeruginosa* suspended in nutrient agar, as reported in a previous paper.[3] Data are presented to show that under the conditions of these experiments, lysis of extracellular DNA by SD is more important than fibrinolysis in the dissolution of the exudate.

METHODS

Enzyme preparations.—SK and SD are prepared commercially from broth cultures of group C streptococci. Both enzymes are excreted into the medium by the bacterial cells, from which they are separated by Seitz filtration. The sterile broth filtrate is then concentrated and dried by lyophilization. Such preparations are stable for considerable periods of time in the dry state. SD may be obtained in relatively pure form by fractional precipitation of the SK with 20% ethanol or 50% ammonium sulfate at pH 5.6.[4] Preparations of SK and SD contain varying amounts of hyaluronidase, ribonuclease and several nucleosidases and nucleotidases as contaminants.[1]

The following preparations were employed in these studies: (1) SK, 100,000 units, and SD, "at least 25,000 units" per vial, lot #2200–537A, supplied by Lederle Laboratories, through the courtesy of Dr. J. M. Ruegsegger; (2) "purified" SD containing 100,000 units per vial but no SK, lot #7-5246, supplied by Lederle Laboratories; (3) a preparation of partially purified plasmin obtained 3 years previously from Dr. L. R. Christensen, and tested for its clot-lysing ability

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1. Tillett, W. S. 1949–50, Studies on the enzymatic lysis of fibrin and inflammatory exudates by products of hemolytic streptococci. The Harvey Lectures, Springfield, Ill., Charles C Thomas, pp. 149–210.
2. Sherry, S., Tillett, W. S. and Christensen, L. R., 1948, Presence and significance of desoxyribose nucleoprotein in the purulent pleural exudates of patients. Proc. Soc. Exper. Biol. & Med. **68**: 179–184.
3. Hamburger, M., Clark, K. L., Jervey, L. P. and Biehl, J. P. 1955, Studies in experimental meningitis in rhesus monkeys. I. The pathogenic effect of various bacteria recovered from human cases. J. Infect. Dis. **97**: 39–47.
4. Christensen, L. R., 1949, Methods for measuring the activity of components of the streptococcal fibrinolytic system, and streptococcal desoxyribonuclease. J. Clin. Investigation, **28**: 163–172.

by Dr. Sol Sherry at the time of these experiments.

Procedure.—The method used, with minor variations which will be described, was essentially the same for all the experiments reported. Healthy *Macaca mulatta* monkeys weighing approximately 8 pounds were infected by the intracisternal injection of 1 ml of a 10^{-6} dilution of the Kreich strain of *Ps. aeruginosa* suspended in a mixture of nutrient agar and yeast beef broth.[3] The animals were sacrificed on the 5th or 6th day postinoculation and the brains and upper spinal cord removed. Variable amounts of a thick, tough, gray-yellow exudate had developed about the brain stem and cervical cord. The exudate from one or two such monkeys was used in each experiment. It was carefully stripped from the underlying brain and cord, placed in a Petri dish and thoroughly chopped and mixed. The pooled exudate was then divided equally into the appropriate number of test tubes and various solutions added as described below.

Following the addition of the solutions, the tubes were sealed, inverted gently to insure mixing, and placed in a constant temperature water bath at 36 to 37 C, where a continuous slow rocking motion was maintained. Fifteen minutes, 1 hour and 4 hours later, the tubes were removed briefly for estimation of the turbidity of the supernatant solution, which was expressed grossly as 0 to 4+.

At the end of the 4-hour period the tubes were removed from the bath, centrifuged and the supernatant discarded. The remaining exudate was washed twice with saline, centrifuged after each washing and finally fixed in neutral reagent grade buffered formalin. Sections were subsequently made and stained with hematoxylin and eosin and by the Feulgen technique. The microscopic sections were studied independently and without knowledge as to whether they were controls or enzyme-treated specimens.

Assays for dornase activity were performed by the viscosimetric method of McCarty,[5] modified according to suggestions made by Dr. Sol Sherry[6] for increasing the sensitivity. The substrate was 0.15% DNA* in 0.003 *M* $MgSO_4$ solution. The enzyme standards and the solutions for testing were diluted in *M*/40 veronal buffer at pH 7.5, containing 2% neopeptone. Specimens were tested undiluted first, and dilutions prepared when necessary. Ostwald viscosimeters were used, with a capacity of 2.5 ml and flow times for water between 8 and 10 seconds. The viscosimeters were placed in a constant temperature water bath at 37 C. Then 0.1 ml. of the buffer solution containing spinal fluid or enzyme solution was added to 2.4 ml of the DNA solution and the solution mixed. The first reading was made 1 minute after mixing and subsequent readings at 1- or 2-minute intervals.

Under these conditions 1 unit of SD is the amount of enzyme contained in 0.1 ml of solution which will cause a drop of 1 viscosity unit in 10 minutes. The DNA solutions were checked frequently against fresh standard enzyme solutions.

The method of Christensen[4] was used for plasminogen determinations.

5. McCarty, 1946, Purification and properties of desoxyribonuclease isolated from beef pancreas. J. Gen. Physiol. **29**: 123–139.
6. Sherry, S. Personal communication.

* Kindly supplied by Dr. J. M. Ruegsegger of Lederle Laboratories.

EXPERIMENTAL RESULTS

Effect of SK-SD dissolved in saline, SK-SD dissolved in serum, and serum alone.—Duplicate experiments were performed 2 months apart. The exudate from 2 monkeys was divided into 5 approximately equal parts. Solutions were added to the tubes as follows:

Tube I—normal saline 5 ml.

Tube II—SK and SD dissolved in 5 ml normal saline. The final concentrations were: SK, 2000 units, and SD, 500 units per ml.

Tube III—normal saline 1 ml; pooled monkey serum 4 ml.

Tube IV—pooled monkey serum 4 ml; SK and SD in 1 ml normal saline. The final concentrations of SK and SD were the same as in Tube II.

Tube V—formalin, 5 ml.

The results of the in vitro digestion in tubes I to IV are shown in table 1. Tube V served as a control for the histologic studies and was not placed in the water bath.

It will be observed that tubes which contained enzymes (II and IV) showed

TABLE 1.—*Effect of SK-SD and SK-SD plus monkey serum upon meningeal exudate in vitro.*

	I (Saline)	II (Saline + SK-SD)	III (Saline + serum)	IV (Serum + SK-SD)
15 minutes	0	++	0	+
1 hour	0	+++	0	+
4 hours	+	++++	+	++++

0 to ++++ indicates turbidity of supernatant after various periods of incubation.
The weight of the exudate varied from 0.094 to 0.154 g.

an increase in supernatant turbidity, whereas the control tubes (I and III) showed little or no change. However, tube IV, which contained SK-activated plasminogen, showed no increase in turbidity over tube II, which contained SK and SD but no plasminogen. The tube containing serum plus saline showed no increase in turbidity whatever. These experiments suggested that the increase in turbidity in the supernatant was associated with the presence of SD rather than with fibrinolytic activity.

In an effort to separate the dornase effect from the fibrinolytic effect the following experiment was carried out. The exudate from 2 rhesus monkeys was collected and divided into 6 approximately equal parts. Solutions were added to the tubes as follows:

Tube I—normal saline, 5 ml.
Tube II—SK and SD in 5 ml normal saline. The final concentrations were: SK, 2000 units and SD, 500 units per ml.
Tube III—normal saline 5 ml; partially purified plasmin 10 mg (2 mg per ml).
Tube IV—partially purified SD (2500 units) in 5 ml normal saline, giving a final concentration of 500 units per ml.
Tube V—partially purified SD (2500 units) and partially purified plasmin 10 mg in 5 ml normal saline, giving a final concentration of 500 units SD and 2 mg plasmin per ml.
Tube VI—formalin, 5 ml.

The results of in vitro digestion in tubes I to V are shown in table 2. Tube VI served as a control for histologic studies and was not placed in the water bath.

TABLE 2.—*Effect of SK-SD, SD alone, "plasmin" alone and SD plus plasmin upon meningeal exudate in vitro.*

	I (Saline)	II (Saline + SK-SD)	III (Saline + plasmin)	IV (Saline +SD)	V (Saline +SD + plasmin)
15 minutes	0	++	+	++	++
1 hour	0	+++	+	+++	++
4 hours	+	++++	+	++++	+++

The weight of the exudate varied from 0.256 to 0.313 g.

Only those tubes which contained SD (II, IV, V) showed an increase in turbidity. The tube containing partially purified plasmin alone (III) showed essentially no change.

Pooled serum from 6 monkeys was used in these experiments. That used in the first experiment contained 2500 units of plasminogen per ml. The plasminogen content of the serum was not specifically determined for each of the other experiments, but random determinations on pooled monkey serums showed concentrations of 800 to 2500 units per ml.

Histologic examination of the digested exudate.—Histologic examination of the exudate remaining after digestion revealed an absence or very marked diminution in the amount of extracellular Feulgen-positive material (DNA) in all specimens which had been exposed to solutions containing SD, as compared with those not containing this enzyme. No difference in fibrin content could be detected between control tubes and tubes containing plasmin or SK plus serum. A detailed description of the microscopic appearance of the exudate was given in the first paper of the series.

Quantitative effect of SD on the exudate.—In view of the striking effect of SD upon the exudate, it became desirable to know how small a concentration of the enzyme would be required to effect digestion. Accordingly, the pooled exudate from 2 infected untreated monkeys was divided equally into 6 tubes, and the following solutions of partially purified SD in 5 ml normal saline added:

Tube I—200 units per ml.
Tube II—100 units per ml.
Tube III—20 units per ml.
Tube IV—2 units per ml.
Tube V—normal saline, 5 ml.
Tube VI—formalin, 5 ml.

Tube VI served as the control for histologic studies and was not placed in the water bath.

TABLE 3.—*Quantitative effect of SD upon meningeal exudate in vitro.*

	I (200u/ml)	II (100u/ml)	III (20u/ml)	IV (2u/ml)	V (Saline)
15 minutes	+	+	0	0	0
1 hour	+++	+++	++	+	0
4 hours	++++	++++	+++ to ++++	++ to +++	+

Weight of the exudate varied from 0.400 to 0.459 g.
0 to ++++ indicates turbidity of the supernatant after various periods of incubation.

The results of this experiment are shown in table 3. It is apparent that under these conditions the smallest concentration of SD required to produce maximum digestion was somewhere between 20 and 100 units per ml, although with as low a concentration as 2 units per ml a significant increase in turbidity occurred.

Effect of SD upon the exudate in situ.—In this experiment an attempt was made to approximate more closely the conditions existing in the living infected monkey when treated with enzymes. For this purpose it was decided to immerse the exudate-bearing portion of a brain in the enzyme solution to note whether or not digestion occurred. One monkey was infected and sacrificed on the 5th day. Inspection of the brain revealed the presence of scanty exudate over the ventral surface of the medulla. The medulla was dissected away from the remainder of the brain stem, and then cut into 2 portions so as to divide the exudate equally. Several slices were made through the arachnoid in each segment with the scalpel to insure contact with the solutions. Each half was then placed in a 50 ml test tube and the following solutions added:

Tube I—normal saline, 9 ml; merthiolate* (1:100), 1 ml.

Tube II—merthiolate (1:100), 1 ml; SK and SD in 9 ml normal saline. The final concentrations were: SK, 2000 units and SD, 500 units per ml.

The tubes were then stoppered, sealed with paraffin and placed in the water bath in the same manner as in previous experiments, a constant rocking motion being maintained. After 18 hours of incubation, the tubes were removed, the appearance of the supernatant noted and the exudate carefully inspected for any evidence of dissolution. Each half was then fixed in Zenker's solution and sections subsequently taken for histologic examination.

At the end of the 18 hours, there was no detectable gross change in the amount or consistency of the exudate from either tube. Each showed a slight increase in the cloudiness of the supernatant. However, microscopic examination of stained sections revealed an almost total absence of extracellular Feulgen positive material in the tissue from tube II as compared to that from tube I. There was no apparent effect on the fibrin in the exudate in either tube.

Lack of effect of SD upon agar inoculum.—In order to rule out the possibility that the turbidity changes might be due to digestion of inoculum rather than exudate the following experiment was carried out.

The pseudomonas-agar inoculum was prepared in the usual manner, and allowed to gel. It was then cut into small blocks of approximately 75 mg each. The material was placed in 5 test tubes to which the following solutions were added:

Tube I—normal saline, 5 ml.

Tube II—SK and SD in 5 ml normal saline. Final concentrations: SK, 2000 units and SD, 500 units per ml.

* Merthiolate was used to diminish secondary bacterial growth during the period of incubation.

Tube III—pooled monkey serum, 4 ml; SK and SD in 1 ml normal saline. Final concentrations: SK, 2000 units and SD, 500 units per ml.

Tube IV—pooled monkey serum, 4 ml; SK and SD in 1 ml normal saline. Final concentrations: SK, 20,000 units and SD, 500 units per ml.

Tube V—partially purified SD, 100,000 units in 5 ml normal saline (20,000 units per ml).

The remainder of the experiment was conducted in the same manner as those previously described. Readings of supernatant turbidity were made at 15 minutes, 1 hour and 4 hours.

At the end of 4 hours there had been no change in the supernatant in any tube nor were any of the blocks visibly affected by the solutions.

Failure of pseudomonas-agar inoculum to alter potency of SD.—The effect of the agar inoculum upon the potency of SD was also determined. Twenty thousand units of SD were incubated for 24 hours with 1 ml of the inoculum prepared in the usual manner, and 20,000 units incubated with saline as a control. At the end of this period, aliquots were removed from each tube and the enzyme content of each determined by its ability to reduce the viscosity of a standard solution of DNA. No significant reduction in enzyme potency occurred in the tube containing the inoculum.

Lack of effect of nutrient agar inoculum on SK.—To make certain that the absence of fibrinolytic activity was not due to inhibition of some component of the fibrinolytic system by the nutrient agar inoculum, an additional experiment was performed. A modification, developed by Glueck,[7] of the fibrin-plate technique of Astrup[8] for fibrinolytic assay was used. In the modified method the substrate is human or bovine fibrinogen suspended in agar. The suspension is allowed to gel in a Petri dish and filter-paper discs (Schleicher and Schuell no. 740–E) are placed on the surface of the plate. Serial 10-fold dilutions of the solution for assay (in these experiments human serum mixed with SK-SD) are pipetted onto the discs and the Petri dish is then incubated for 24 hours at 37 C. The plates are smoky in appearance, and lysis of the fibrinogen is shown by an area of clearing around the disc.

Plates made with nutrient agar and both human and bovine fibrinogen were made and observed simultaneously with those made from plain agar. Zones of clearing on the nutrient agar-fibrinogen plates correlated closely with those found on the plain agar-fibrinogen plates, indicating that nutrient agar does not interfere with the fibrinolytic system.

DISCUSSION

The experiments show clearly that in the pyogenic experimental meningitis under investigation, extracellular DNA is an important constituent of the exudate; the DNA is susceptible in vitro to the action of SD in as low a concentration as 2 units per ml; and under the conditions of these experiments, the fibrin present was not demonstrably affected by plasmin. These findings are in contrast with those of Fletcher[9] and Cathie[10] in tuberculous meningitis, where fibrin is apparently the chief component of the exudate, and where lysis of the exudate, in vitro at least, can be achieved with plasmin.

7. Glueck, H. I. Personal communication.
8. Astrup T. and Mullerty, S. 1952, The fibrin plate method for estimating fibrinolytic activity. Arch. Biochem. & Biophysics, **40**: 346–351.
9. Fletcher, A. P. 1954, Intrathecal fibrinolysis with streptokinase in tuberculous meningitis. J. Clin. Investigation, **33**: 69–76.
10. Cathie, I. A. B. 1949, Bacterial fibrinolysin, its possible therapeutic application in tuberculous meningitis. J. Clin. Path. **2**: 73–76.

Sherry and Tillett,[3,11] however, have demonstrated the importance of SD in the enzymatic treatment of empyema thoracis where 30 to 70% of the total solid material consists of desoxyribose nucleoprotein. In contrast to tuberculous meningitis, and in similarity with empyema thoracis, the exudate in the experimental meningitis is almost completely polymorphonuclear in cell type and contains large amounts of extracellular Feulgen-positive material (DNA). It seemed logical that SD would play a significant role in the lysis of such an exudate.

The experiments summarized in table 1 showed that lysis of the exudate occurred when SK-SD solutions were added, but they also indicated that SK is not necessary to produce this effect. They did not completely eliminate SK, since the remote possibility existed that plasminogen present in the exudate itself may have been activated by the SK. However, microscopic examination of the exudate remaining after digestion showed no reduction in the amount of fibrin present. Further experiments demonstrated that SD alone was responsible for the lysis, since only tubes containing SD, whether by itself or in combination with SK, showed evidence of lysis. Furthermore, the exudate from tubes containing plasmin with SD, or SK-SD with serum of relatively high plasminogen content (800 to 2500 units per ml), showed no evidence of fibrinolysis but did show almost complete dissolution of extracellular DNA in the microscopic sections of the digested exudate. Exudate from tubes containing plasmin alone showed no differences from the control on gross or microscopic examination.

11. Sherry, S., Johnson, A., and Tillett, W. S., 1949, The action of streptococcal desoxyribose nuclease (streptodornase) in vitro and on purulent pleural exudations in patients. J. Clin. Investigation, **28**: 1094–1104.

The data in table 3 suggest that the minimum level of dornase activity which will produce effective lysis of the exudate in 4 hours is in the neighborhood of 2 units per ml. At this concentration lysis does not begin as early as with levels of 20 units per ml or greater. The results suggest that the optimum range may lie between 2 and 20 units per ml. Data presented in the following paper indicate that such levels in the spinal fluid are in fact associated with demonstrable effects in vivo.

SUMMARY AND CONCLUSIONS

1. Purulent exudate scraped from the meninges of monkeys experimentally infected with *Pseudomonas aeruginosa* suspended in agar was incubated with various solutions containing streptokinase-streptodornase plasmin, and SD. The effect of these enzyme solutions was measured by turbidity changes in the supernatant fluid and by changes in the microscopic picture of the exudate after exposure to the solutions.

2. As judged by turbidity changes and by the disappearance of extracellular DNA from the microscopic preparations of the exudate, SD is effective in vitro in the lysis of the exudate of experimental purulent meningitis. Concentrations of the enzyme as low as 2 units per ml are effective in producing lysis. The range for optimum activity appears to lie between 2 and 20 units per ml.

3. SK, SK plus serum, and a preparation of partially purified plasmin produced no detectable gross change in the exudate, and microscopic studies did not reveal any effect on its fibrin content.

4. The agar vehicle used in the inoculum was not affected by SD and had no effect on the action of SD.

STUDIES IN EXPERIMENTAL MENINGITIS IN RHESUS MONKEYS

III. THE EFFECT OF INTRACISTERNAL INJECTION OF STREPTOKINASE AND STREPTODORNASE ON THE EXUDATE OF EXPERIMENTAL MENINGITIS

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The purpose of the investigations reported in this paper was to study the effect of the intracisternal injection of streptokinase (SK) and streptodornase (SD) on the exudate of experimental *Pseudomonas aeruginosa* meningitis in the rhesus monkey. The original work of Tillett, Christensen, and Sherry[1] focused attention upon the nature of exudates in general, and of pleural exudates in particular. The demonstration by these investigators that SK and SD can often be valuable adjuncts in the treatment of purulent pleural exudates[2,3] aroused interest in the possibility of their use in purulent infections in body spaces other than the pleural cavity. The exudate found in the subarachnoid space in infections of the meninges sometimes creates problems in the successful treatment of clinical meningitis. Such exudate may at times produce a block in the cerebrospinal fluid system, and treatment failures have occasionally been attributed to the persistence of exudate in spite of apparently adequate antibiotic therapy.

Studies of the effect in vitro of SK and SD on the exudate of experimental meningitis were reported in a previous paper.[4] These studies showed definite lysis of extracellular desoxyribonucleic acid (DNA) by SD, but failed to show any evidence of fibrinolysis resulting from the use of either plasmin or SK with monkey serum. The experiments reported here were undertaken in an effort to determine the effect of SK and SD on meningeal exudate in the intact animal.

METHODS

Meningitis was produced in 6 to 9 pound *Macaca mulatta* monkeys by the intracisternal inoculation of *Ps. aeruginosa* (Kreich strain) suspended in nutrient agar.[5] The actual number of

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1. Sherry, S., Tillett, W. S., and Christensen, L. R. 1948, Presence and significance of desoxyribose nucleoprotein in the purulent pleural exudates of patients. Proc. Soc. Exper. Biol. & Med. **68**: 179–184.
2. Tillett, W. S. and Sherry, S. 1949, The effect in patients of streptococcal fibrinolysin (streptokinase) and streptococcal desoxyribonuclease on fibrinous, purulent and sanguinous pleural exudations. J. Clin. Investigation, **28**: 173–189.
3. Sherry, S., Johnson, A. and Tillett, W. S. 1949, The action of streptococcal desoxyribose nuclease (streptodornase) in vitro and on purulent pleural exudations in patients. J. Clin. Investigation, **28**: 1094–1104.
4. Jervey, L. P., Clark, K. L., Freiman, D. G. and Hamburger, M. 1956, Studies in experimental meningitis in rhesus monkeys. II. The importance of streptodornase in the enzymatic digestion in vitro of experimentally produced meningeal exudate. J. Infect. Dis. **97**: 300–304.
5. Hamburger, M., Clark, K. L., Jervey, L. P.,

organisms injected varied between 252 and 5540. The larger inocula were required in the later experiments in order to produce the same amount of exudate in the control animals as that obtained in the earlier experiments. Repeated subarachnoid passage of the *Pseudomonas* was necessary to maintain the virulence of the strain for the meninges.

Cisternal punctures were done at 24-hour intervals after the introduction of the infecting inoculum, and fluid was removed whenever possible. Leucocyte counts, Pandy test, smear, culture and assays for plasmin and desoxyribonuclease (dornase) activity were performed if enough fluid was obtained. Following the aspiration of fluid, and with the needle still in place, solutions of SK-SD,* lots #7-1089-121A and #7-1089-232A, or of SD* alone, lot #7-5246, were injected into the cistern. This was done on days 2, 3, and 4. On day 5 a diagnostic cisternal puncture was done and the monkey sacrificed.

The monkeys were divided into 3 groups. One group received no treatment other than daily aspiration of cisternal fluid. Another received 1 ml of 0.85% saline into the cistern on days 2, 3, and 4. The 3rd group comprised the enzyme-treated monkeys. The enzyme solutions, always in a volume of 1 ml., contained 25,000 to 26,000 units of SK plus 4320 to 6250 units of SD, or 100,000 units of SD alone. In many instances in which cerebrospinal fluid could not be obtained by cisternal puncture, the solutions were injected blindly after the needle was thought to have penetrated the dura. The needle was considered to have been correctly positioned if cloudy or purulent fluid could then be withdrawn and reinjected easily. This was usually possible.

Specimens of fluid for assay of plasmin and dornase activity were centrifuged for 30 minutes at 2000 r.p.m. and stored at −15 C until the determinations were made. Plasmin time was determined by the method of Christensen.[6] Dornase activity was determined by a modification of the viscosimetric method of McCarty described previously.[4]

Cultures of spinal fluid and exudate were made in yeast-beef broth and on yeast-beef agar. Gram-negative rods recovered from these cultures were identified as *Ps. aeruginosa* by colony appearance, liquefaction of gelatin and green pigment production (or green fluorescence under ultraviolet light). Further identification of the "Kreich" strain was accomplished by measurement of the antibiotic spectrum, using a two-fold serial dilution test. Expressed as mg per ml., the minimal inhibiting concentrations were as follows: chloramphenicol, 125, chlortetracycline, 31.2; oxytetracycline, 15.6; streptomycin, 15.6; and polymyxin B, 0.78. A variation of one dilution in either direction was considered acceptable.

The exudate was examined at necropsy and graded as to degree. The investigator grading the exudate was unaware of the type of treatment the animal had received, if any. "None" indicated the absence of grossly visible exudate. "Scanty" represented definite clouding and dullness of the meninges. "Moderate" indicated opacity of the meninges with at least a small amount of free exudate which could be wiped off. "Profuse" indicated abundant thick layers of free exudate.

Microscopic studies were made on sections stained by the Feulgen method, the Mallory phosphotungstic acid stain, and hematoxylin and eosin. The microscopic sections were studied independently and without knowledge of the treatment received by the particular monkey or of the gross necropsy findings. Results of the gross and microscopic studies were correlated after their independent completion. Strict quantitation of extracellular DNA and of fibrin was not possible, since the amount of these substances varied in different parts of the exudate. Semiquantitative estimates could be made, however, by examination of different sections of the exudate.

RESULTS

Effect of SK-SD, SD and saline upon the gross and microscopic appearance of the exudate.—Autopsy estimates of the gross quantity of meningeal exudate in the treated and control monkeys are presented in tables 1 and 2. As shown in table 1, there was no significant difference between SK-SD treated and saline treated animals, although both groups when compared with the controls showed approximately a 48% reduction in the number of animals displaying profuse exudate, with a parallel increase in the number showing scanty to moderate exudate.

As shown in table 2, the results were

and Biehl, J. P. 1955, Studies in experimental meningitis in rhesus monkeys. I. The pathogenic effect of various bacteria recovered from human cases. J. Infect. Dis. **97**: 39–47.

* Supplied by Lederle Laboratories through the courtesy of Dr. J. M. Ruegsegger.

6. Christensen, L. R. 1949, Methods for measuring the activity of components of the streptococcal fibrinolytic system, and streptococcal desoxyribonuclease. J. Clin. Investigation, **28**: 163–172.

TABLE 1.—*Effect of daily intracisternal injections of SK-SD solutions upon the amount of gross exudate found in the subarachnoid space of monkeys infected with* Ps. aeruginosa *suspended in agar.*

Intracisternal treatment	Number of monkeys	Number of animals showing exudate			
		None	Scanty	Moderate	Profuse
None	7	0	0	0	7
Saline	15	0	4	3	8
SK-SD	21	1	6	3	11

Doses: 25,000–26,000 units SK plus 4320–6250 units SD. All control monkeys ("none" and "saline") were studied simultaneously with the SK-SD treated monkeys.

somewhat different when the daily dose of SD was increased from approximately 5000 to 100,000 units. The percent of animals showing profuse exudate was greatest in the control group, next in the saline group, and least in the SD-treated group. These differences are suggestive, but because of the relatively small numbers of animals involved, are not statistically significant.

Microscopic estimates of the total amount of exudate—slight, moderate or profuse—agreed closely with gross estimates and indicated representative sampling in the large majority of instances. Estimates were also made of the amount of partially depolymerized extracellular desoxyribosenucleoprotein in the exudate, as evidenced by the basophilia with hematoxylin and by the positive Feulgen reaction. Estimates of the amount of fibrin were based on the routine sections and in many instances on a corresponding block fixed in Zenker's fluid and stained by the Mallory phosphotungstic acid hematoxylin method. It was soon found that a good general estimate could be made based on the combined findings of several sections stained with hematoxylin and eosin alone. Special stains were therefore used only for confirmation in selected instances.

TABLE 2.—*Effect of daily intracisternal injection of 100,000 units "purified" SD solutions upon the amount of gross exudate found in the subarachnoid space of monkeys infected with* Ps. aeruginosa *suspended in agar.*

Intracisternal treatment	Number of monkeys	Number of animals showing exudate			
		None	Scanty	Moderate	Profuse
None	8	0	1	1	6
Saline	8	0	3	2	3
SD	12	0	5	6	1

Monkeys were treated on the 2nd, 3rd and 4th days and sacrificed on the 5th day. All control monkeys ("none" and "saline") were studied simultaneously with the SD treated monkeys.

In the untreated control group, the amount of extracellular DNA was striking and was graded as moderate or profuse in 11 of 15 cases, 5 of these being classed as profuse. The saline treated group showed little significant difference from the controls, with 10 of 17 cases being graded as moderate or profuse, 6 of these being considered profuse. In the SK-SD treated group, however, only 4 of 18 cases could be classed as moderate and none as profuse, indicating a sharp and significant decrease in the amount of extracellular DNA present. A similar though less striking change was noted in the groups treated with SD alone, with 6 of 10 cases showing moderate amounts of extracellular DNA and none showing more than this. The fibrin and cellular contents of the exudate, on the other hand, were essentially similar in all groups including those receiving SK.

Assay of spinal fluid for fibrinolytic activity (plasmin).—Plasmin assays were performed on 25 samples of spinal fluid from untreated monkeys and from animals 24 hours after intracisternal injections of SK-SD, or 24 hours after saline. The difficulty of obtaining sufficient fluid for testing made a more extensive investigation impossible. Amounts of fluid adequate to permit plasminogen as well as plasmin assays could not be obtained.

The essentially negative results of the plasmin assays are shown in table 3. Trace to 1+ activity was present in 10 out of 12 samples obtained before infection. In only 1 instance was significant

TABLE 3.—*Plasmin assays on cerebrospinal fluid of control monkeys and of monkeys treated with intracisternal injections of SK-SD.*

Monkey no.	Day 1	2	3	4	5	Treatment
8	Tr.	0				None
5	Tr.					None
4	Tr.					None
2	+					None
9	0					None
18	+	0	0			NaCl
7	Tr.					NaCl
3	Tr.	+		0		SK-SD*
12	Tr.		0	0	0	SK-SD*
13	Tr.		3+		0	SK-SD*
17	0				0	SK-SD*
16	Tr.	0	0			SK-SD*

* Dose: 26,000 units SK, 4320 units SD, given on days 2, 3, and 4.
Plasmin activity expressed as:
4+ = lysis of standard clot in 1 hour or less
3+ = lysis in one to 6 hours
2+ = lysis in 6 to 24 hours
1+ = lysis in 24 to 48 hours
Tr. = lysis in 48 to 72 hours, but no lysis in controls
0 = no lysis

(3+) plasmin activity obtained after the production of meningitis and this occurred in an SK-SD treated monkey.

Because of the failure to find fibrinolytic activity 24 hours after the injection of SK, an experiment was carried out in an attempt to demonstrate such activity after 4 hours. Two infected monkeys were treated with 26,000 units SK and 4320 units SD and 2 with 1 ml of saline. Fluid was obtained on day 1, prior to infection. On days 2, 3, and 4 cisternal punctures were performed at 10:00 a.m. and, if possible, fluid was withdrawn for assay. Then saline or SK-SD solution was injected. Cisternal punctures were repeated 4 hours later, samples were again withdrawn, and another injection of saline or SK-SD solution made. This procedure provided plasmin assays at 4, 20, and 24 hours following the injections of SK-SD.

No fibrinolytic activity was found in any of the 18 specimens studied. They included 4 fluids drawn prior to infection, 1 after infection but before treatment, 4 samples 4 hours after SK-SD, 2 20 hours after SK-SD, and 7 at 4- or 20-hour intervals after intracisternal injection of saline. Plasminogen assays on blood drawn from these 4 monkeys showed 800 units per ml in 1, and 1600 units per ml in the remaining 3.

Thus, in spite of adequate amounts of plasminogen in the blood, no significant fibrinolytic activity was demonstrable in the spinal fluid, either with or without the use of SK.

Assays of spinal fluid for dornase activity.—Dornase activity was studied in 6 infected monkeys given 100,000 units of SD at 24-hour intervals, on days 2, 3, and 4 (table 4). Fluids from the 6 monkeys were negative prior to treatment. Thirteen of 14 samples obtained on days 3, 4, and 5 showed levels varying from 2 to 100 units per ml. One specimen obtained on day 5 showed no activity. No toxic effects from this dose were found in infected monkeys. A positive Pandy test and elevated spinal fluid leucocyte counts were produced in 2 normal monkeys. The leucocyte counts 3 hours after the intracisternal injection were 1900 and 36,000 per mm^3; at 24 hours, 13,000 and 20,000; at 48 hours, 3400 and 2560; and at 120 hours, 284 and 1692, respectively.

The persistence of dornase activity in the cerebrospinal fluid of 2 uninfected monkeys was studied following a single intracisternal injection of 100,000 units of SD. No activity was found in the spinal fluid prior to injection of SD. Three hours after injection, 2000 units per ml were present in both animals. At 24 hours, 100 units per ml were found

TABLE 4.—*Cerebrospinal fluid levels of SD in infected monkeys given intracisternal injections of 100,000 units SD on days 2, 3, and 4.*

Monkey no.	SD per ml CSF on day: Pre-treatment	3	4	5
110	0			0
117	0	>10*		50
118	0	6	10	4
121	0	100	5	3
116	0	2		>10*
120	0	7	100	5

Day of infection is considered day 1.
* Insufficient fluid to permit further dilution.

in one monkey, and 3 units in the other. At 48 hours only one animal still showed 2 units. This was the monkey with the highest level (100 units per ml) on the preceding day. No activity was detected after 48 hours.

Cultures.—Spinal fluid cultures were obtained 24 hours after infection from 66 of the 75 animals. All 66 were positive for the Kreich strain of *Ps. aeruginosa*. A total of 139 cultures was obtained on days 3, 4, and 5. Of these, 136 were positive. One monkey treated with SK-SD had negative cultures on days 4 and 5. Post mortem cultures of the exudate were contaminated. One monkey given SD intracisternally had a negative culture on day 4, but culture of the exudate at necropsy yielded *Ps. aeruginosa*.

Leucocyte counts.—The mean daily leucocyte count of the spinal fluid was quite high in both treated and control animals. All but 4 of 121 counts done on days 2, 3, 4, and 5 were in excess of 1000 per mm^3. Nineteen counts were in excess of 50,000 and 14 of these were greater than 100,000 per mm^3. The wide range of the counts in all animals made it impossible to demonstrate significant differences between control and treated animals.

Pandy test.—The Pandy test after day 1 was strongly positive in 155 out of a total of 156 determinations.

Mortality.—Inasmuch as few animals died within the 5 day study period of the experiments, no effort was made to assess the comparative mortality of treated and untreated animals. The few animals which died were fairly uniformly distributed among treated and control monkeys and death could usually be ascribed to accidental injury to the medulla, to anesthesia or to subarachnoid hemorrhage.

DISCUSSION

These experiments demonstrate that the injection of SD into the cisterna magna of monkeys experimentally infected with *Ps. aeruginosa* reduced the amount of extracellular DNA in the exudate. The effects upon the gross appearance of the exudate were less striking. When doses of 100,000 units of SD were employed daily, only 1 out of 12 monkeys displayed profuse amounts of exudate in the meninges, as contrasted with 6 of 8 untreated monkeys and 3 of 8 treated with normal saline. When a smaller dose of SD (4200-6200 units) combined with SK was given, the reduction in the gross amount of exudate was the same in both enzyme treated and saline treated animals, but there was a definite diminution in the amount of microscopically identified extracellular DNA in the treated group as contrasted with both untreated control and saline treated monkeys. The suggestion has been made that other enzymes, such as hyaluronidase and ribonuclease, commonly present as contaminants in the SK-SD preparations, and possibily also in the SD preparation, may have contributed to the lysis of the exudate, but we have no evidence to support or reject the possible roles of these enzymes.

Dornase activity persisted in the spinal fluid of treated and untreated animals for at least 24 hours in concentrations shown to be effective in dissolving extracellular DNA in meningeal exudate in vitro.[4] This finding suggests that should SD be used therapeutically it would not be necessary to inject the enzyme more than once daily.

In contrast with the efficacy of SD was the failure of intracisternally injected SK to produce a fibrinolytic effect under the conditions of the experiments. The virtual absence of fibrinolytic activity in the spinal fluid of the infected monkeys could be due to low precursor (plasminogen) content, to inefficient conversion of precursor to

enzyme, to high inhibitor content of the spinal fluid, or to a combination of these factors.[7] The possibility that plasmin is not stable in spinal fluid when frozen and then thawed must be considered as a possible explanation of its absence in the fluids tested. However, the absence of any evidence of fibrinolysis in the microscopic sections of the exudate suggests that there was little if any plasmin in the spinal fluid before freezing.

The amounts of spinal fluid obtainable were not large enough to permit the assay of plasminogen or of plasmin inhibitor in these experiments. Biehl, working in this laboratory, studied plasminogen levels in the spinal fluid of monkeys with experimental meningitis due to *Diplococcus pneumoniae*, type I.[8] He found detectable levels (25 and 34 units per ml) in only 2 of 43 fluids measured 24 hours after inoculation, in 6 of 14 fluids 48 hours after inoculation (16 to 26 units per ml), and 4 of 7 fluids at 72 hours (10 to 26 units). Other investigators have also reported low plasminogen levels in meningitis and in normal spinal fluid.[9,10]

SUMMARY AND CONCLUSIONS

1. Monkeys experimentally infected with *Pseudomonas aeruginosa* were treated with daily intracisternal injections of SK-SD or SD. Intracisternal administration of solutions containing SD in doses of 100,000 units produced a reduction in the amount of gross exudate seen at autopsy. Smaller doses of SD (4000 units) given in combination with SK did not produce any grossly visible change in the amount or type of exudate. At both dosage levels there was a definite reduction in the amount of extracellular Feulgen-positive material seen on microscopic study. This was not accompanied by any change in the cellular characteristics of the exudate.

2. Levels of dornase activity which appear to be significant were produced in both infected and uninfected monkeys by the intracisternal injection of 100,000 units of SD. These levels persisted for 24 hours or more. No toxic effects from such doses were noted in infected monkeys, but moderate increases in spinal fluid leucocyte count and a positive Pandy test were produced in normal monkeys.

3. No gross or microscopic evidence of significant fibrinolytic activity upon the exudate was found following intracisternal injection of 25,000 units SK, and plasmin could not be detected in the spinal fluid of these animals 4, 20, or 24 hours after injection.

4. These studies show that definite changes can be produced in the exudate of an experimental purulent meningitis by the intracisternal administration of SD and that prolonged apparently significant levels of dornase activity in the spinal fluid are obtainable by this method. The findings suggest that SD may be a useful adjunct in the treatment of purulent meningitis.

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THE ASSAY OF VIRULENCE OF COCCIDIOIDES IN WHITE MICE

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The determination of virulence of the fungi causing systemic infections has stimulated considerable interest but no simple method for their measurement has been developed. The organisms produce chronic mycoses with a variety of disease patterns. Indeed, many human infections are asymptomatic. Probably as a corollary of this broad human infection spectrum, the pathogenic sequence in laboratory animals is not as reproducible as it is in most bacterial and viral infections. This and the feeble pathogenicity of many fungi have led to a variety of assay methods.

In lieu of more precise determinations, the most common procedure has been the administration of serially diluted culture suspensions without quantitating the actual number of organisms. Thus, the cumulative number dying or the average day of death of the inoculated as compared with control animals supplied some information regarding the host-fungus relationship, provided a suitable dose was attained. Recently, virulence of *Coccidiodes immitis* and other fungi has been studied by modifying the resistance of the host with X ray, cortisone or mucin,[1–5] by employing a more susceptible host[6] or by unusual routes of inoculation.[6–8] Karrer,[8] in studying the comparative virulence of two strains of *C. immitis*, chose the intracerebral route of infecting mice since his experience indicated that large dosages are required to kill mice by the intraperitoneal route. Employing the sensitive intracerebral route, he was able to determine an LD_{50} of both strains.

All the methods mentioned are plausible tools for characterizing the virulence of an infectious agent. However, there appeared to be a need for a more thorough study of the virulence of each species by a single route, considering the interrrelationships of the factors involved. One approach, used by one of us for many years, has related percent

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mortality to time of death in evaluating the virulence of *C. immitis*. The present report deals with the comparative virulence of *C. immitis* for mice inoculated intraperitoneally.

METHODS

Source of strains.—Three of the strains of *C. immitis*—Silveira, Perry and Mauser—were isolated from human cases of coccidioidomycosis.* Several soil isolates and approximately 45 additional human isolates were included in the multiple strain suspension. All strains were obtained from the collection maintained at the School of Public Health, University of California, Berkeley, California in connection with research supported by the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board.

Preparation of inoculums.—A multiple strain suspension was prepared by scraping 3% malt extract agar plate cultures which had been dried at 35 C. The dry material was suspended in saline by shaking for approximately 6 hours in a flask containing glass beads and filtered through glass wool.

Preparation of the single strain suspensions followed the same procedure, except that glucose yeast extract agar[9] was employed, the dry material was collected in medium porosity alundum extraction thimbles by vacuum and filtering through glass wool was omitted.

To inhibit germination, stock saline suspensions were stored at 4 C. To attain exact dosages, the stock suspensions were adjusted to the desired concentration by the addition of saline. The number of organisms per dose was verified at the time of animal inoculation by preparing 4 to 6 pour plates (6 inch diameter petri dishes) for each of the suspensions estimated to contain 100 or less viable particles per ml. (The particle count of the "100-dose" suspensions actually ranged from 90 to 99 per ml.)

Animal experimentation.—Except where otherwise indicated, 10-week-old male Namru mice[10] in groups of 25 per dose were used throughout. For the comparison of host susceptibility, 10-week-old male mice of an unknown strain, purchased from a commercial jobber ("commercial" mice), were used. Inoculums were given in 1 ml volumes intraperitoneally.

Survivors were sacrificed after 90 days. All mice were examined for gross evidence of lesions and, if infection was not apparent, the lungs, liver and spleen were cultured.

RESULTS

The comparative virulence of 100 viable particles of the 3 strains was determined by cumulative percent mortality as a function of time. Figure 1 showed that the Silveira strain was most virulent in that deaths were produced more rapidly than with the other strains. The slope of the mortality time curve for the Perry strain was intermediate and the Mauser strain was the most gradual. The virulence of these same strains was compared by three other methods, presented in table 1. First was the number of days after inoculation which were required in order that 50% of the mice would die, designated as the LT_{50}. Second was the percentage of animals dead 30 days after inoculation. Third was the number of viable particles calculated[11] to effect 50% killing at 30 days. This number was calculated from

TABLE 1.—*Comparative virulence in mice of three strains of* C. immitis *as demonstrated by three end points.*

Strain	LT_{50}* (constant dosage 100)	Percent dead at 30 days (constant dosage 100)	Median lethal dose at 30 days (variable dosages)
Silveira	17	84	< 10
Perry	35	40	361
Mauser	90	4	>1000

* Time in days after inoculation at which 50% of the mice were dead.

* Silveira strain was isolated from a patient with primary coccidioidal erythema nodosum without dissemination. He was entirely healthy four years later. The Perry strain was isolated from a patient with a fulminating disseminated coccidioidal infection who died within 3 months of the onset. The Mauser strain was isolated from a patient with coccidioidal meningitis who also died.

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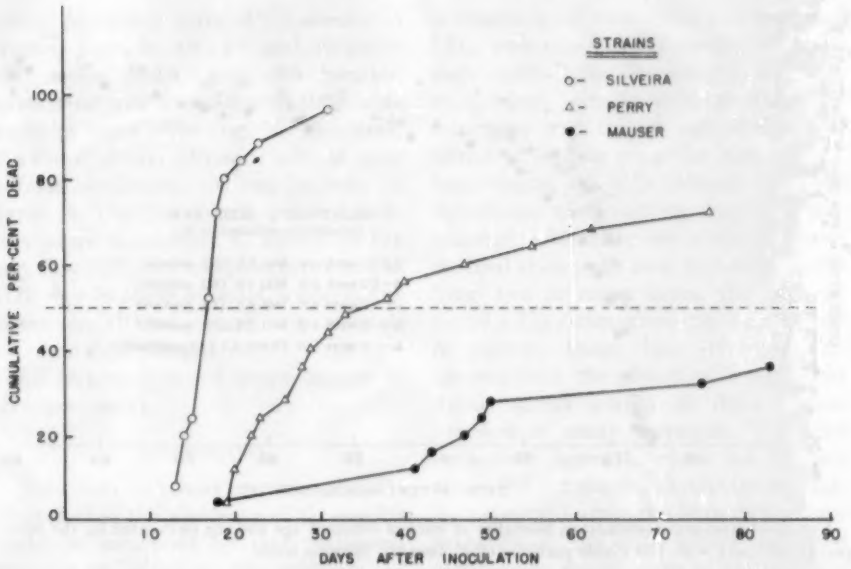

FIG. 1.—Cumulative percentage mortality of mice inoculated with 100 viable particles of three strains of *C. immitis*.

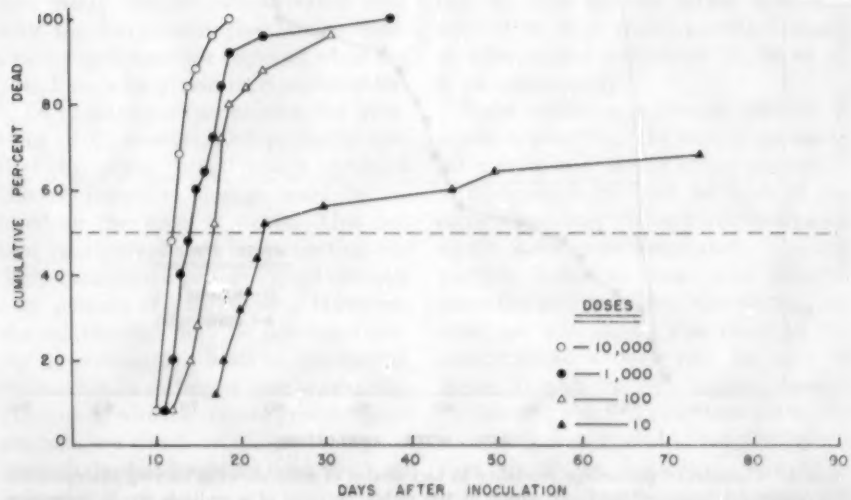

FIG. 2.—Cumulative percentage mortality of mice inoculated intraperitoneally with varying doses of viable *C. immitis* arthrospores, Silveira strain.

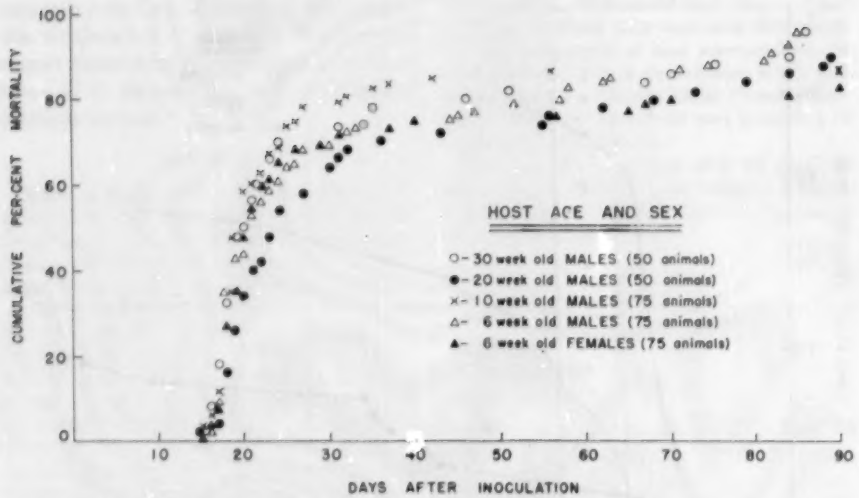

FIG. 3.—Cumulative percentage mortality of mice of different age and sex inoculated by the intraperitoneal route with 100 viable particles of *C. immitis*, Silveira strain.

data including the necessary second dose level. By each of these methods the same relationship of lethality was observed.

Figure 2 shows the dose-mortality relationship in groups of animals inoculated with tenfold decrements of Silveira spores; the greater the dose, the more rapid the killing.

Age and sex did not affect the mor-

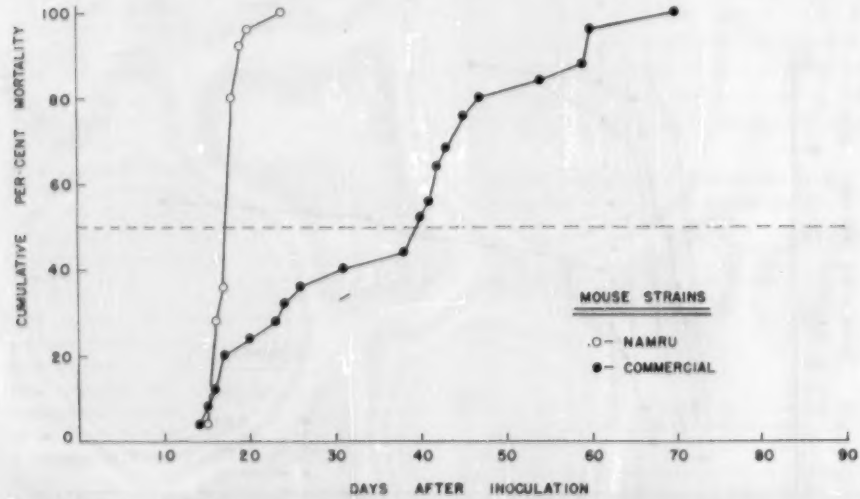

FIG. 4.—Cumulative percentage mortality in two strains of mice showing varying susceptibility with concurrent intraperitoneal inoculation of 100 viable particles of a multiple strain suspension of *C. immitis* arthrospores.

tality. Mortality rates of *C. immitis* in Namru mice, 6-, 10-, 20- and 30-week-old males and 6-week-old females, inoculated concurrently with 100 viable particles were alike (fig. 3). However, the susceptibility of two strains of mice differed strikingly, as can be seen in figure. 4. The Namru mice were markedly more susceptible as shown by the fact that 50% mortality occurred on the 17th day in these animals, while in the "commercial" mice half the animals survived until the 40th day.

All strains infected every mouse in all experiments.

DISCUSSION

The data in these experiments indicated that the virulence of *C. immitis* could be measured by intraperitoneal inoculation without the addition of virulence-enhancing substances. Other studies on the mortality rate of the Silveira strain administered intracerebrally, intravenously and intraperitoneally[12] confirmed the observation of Karrer[8] that the intracerebral route was the most sensitive. However, the present study clearly demonstrated that with these representative strains massive dosages were not required when the inoculum was given intraperitoneally.

To characterize accurately the virulence of *C. immitis*, and probably also that of other fungi which produce chronic infection, dosage must be related to the time of death. This can best be accomplished by inspecting the daily cumulative per cent dead through long periods of observation. However, the relationship may be expressed simply by comparing points on the mortality curves in either of two ways, viz., (1) time at which a certain percentage of animals are dead, or (2) percentage of animals dead at a specific time. Both are expressions of rate. The conventional LD_{50} which requires observing the animals until all that are going to die have succumbed, is most suitable for acute infections and is not appropriate for fungal infections since the latter run a long course. As with chronic bacterial infections, observations may be terminated arbitrarily when deaths have reached their peak and, by using results from two or more doses, the conventional LD_{50} estimations can be adapted to portray these data. However, to assume that the period of most rapid dying occurs within 30 days is unjustified in many instances. This can be seen in figure 1, where the Mauser strain did not show a significant mortality rate until after the 40th day; interpretation of the data before this time erroneously would have minimized the virulence of this strain. That expressions of virulence must be related to the length of observation is further emphasized in the median lethal doses calculated from the mortality following inoculation of varying doses, viz. 1000, 100 and 10 spores, of the Silveira strain (fig. 2). The median lethal dose was 415, 14 or <10 viable particles, based on observation periods of 15, 30 or 45 days, respectively.

Since virulence is always relative, it would appear that the ideal experimental comparison of the lethal properties of *C. immitis* can best be made if the same number of viable particles of each strain have been inoculated. The 100 particle infective dose was selected since the rate of killing was neither too slow nor too rapid. The effect of too concentrated a dose can be seen in figure 2: with the two highest dosages of Silveira, viz., 10,000 and 1000, the mortality was so rapid that discrimination would be fallacious. The three strains of *C. immitis* studied were well suited for this comparative study since

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their lethal dosages were within the 100 particle range. Other strains would require preliminary pilot assays to select the appropriate dosage.

Host factors did not affect infectivity of mice by *C. immitis*. However, although age and sex of mice did not influence virulence (progression) of the established infection, the strain of the mice was important. Thus mice obtained from a commercial source exhibited a very low mortality compared with the high mortality of the Namru strain.

SUMMARY AND CONCLUSIONS

The virulence of three strains of *Coccidioides immitis* have been differentiated in white mice by the intraperitoneal inoculation of 100 viable particles. The host responses have been measured by plotting the daily cumulative percentage of animals dead through 90 days of observation. Variations of virulence have been indicated by markedly differing mortality curves. The same lethal relationships have been expressed by two other indices: (1) length of time in days when 50% of the animals were dead (LT_{50}) and (2) percentage of mice dead at 30 days. Estimations of median lethal dosages have confirmed the same strain relationships when the dimension of time has been fixed. In the measurements of virulence the time of observation must include the period of most rapid dying. Moreover, while dosages must be sufficient to detect lethality, those which are too large will cause such rapid dying that discrimination between strains is impossible.

Host factors have not affected susceptibility to infection. While age and sex have no discernible influence on lethality, two strains of mice have differed markedly in this regard.

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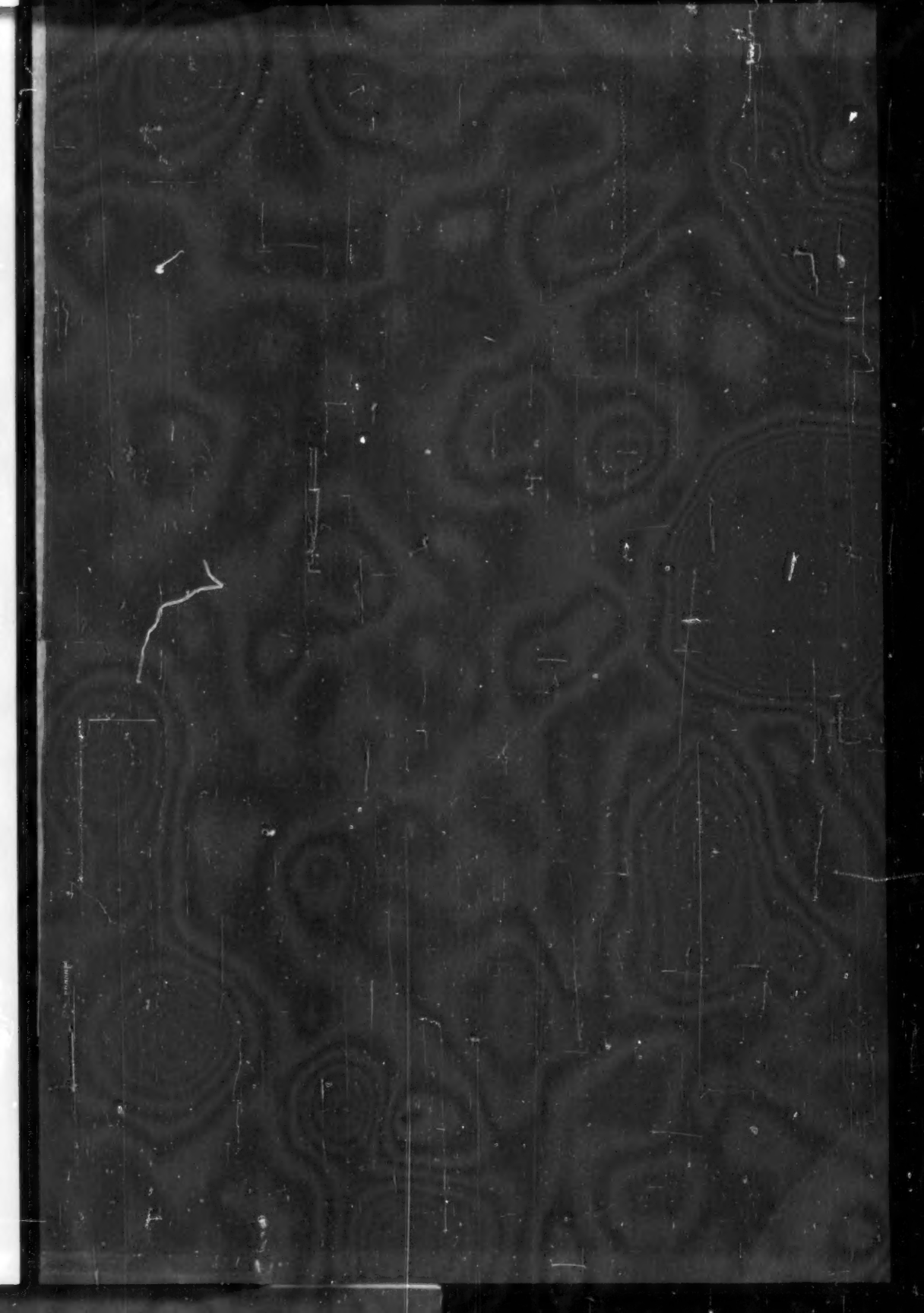

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